ANNUAL REVIEW OF BIOCHEMISTRY

PREFACE

Again we wish to thank most cordially the many who have assisted in the authorship of the present volume. It would be needlessly repetitious to recite, as we have done for several years, the difficulties which have continued to confront our authors, and which indeed have been experienced by all who are engaged in scientific research. We regret that three of the reviews proposed for publication and announced as such in our advance circulars could not be prepared in time for publication: "Nutrition" by B. S. Platt, "The Polynucleotides" by E. Hammarsten, and "The Chemistry and Metabolism of the Compounds of Sulfur" by B. H. Nicolet. Additional space in Volume XVI has been assigned to Professor McHenry in order that the review on "Nutrition" which is to appear in that volume may embrace adequately the work of a two-year period in this very active field.

It is with sorrow that we record the death of our colleague, Professor Carl Schmidt, who from the inception of these *Reviews* has served on the Editorial Committee. His advice has been invaluable, the fruit of years of activity in biochemical research and of a broad acquaintanceship among biochemists both here and abroad.

Notice has already been given of the intent of Annual Reviews, Inc., to publish an *Annual Review of Microbiology,* the first volume of which is scheduled to appear in November 1947. In order to facilitate the preparation of three *Reviews* a change in publication schedule has been made: commencing in 1947 the *Annual Reviews of Physiology, Biochemistry,* and *Microbiology* will be released from the press in March, July, and November, respectively.

To H. A. Barker, H. J. Deuel, and Hans Lineweaver we are greatly indebted for their kindness in participating in the meeting of the Editorial Committee at which the content and authorship of the present volume were determined. As of this date we must record with regret the termination of service as associate editor of Dr. J. H. C. Smith, who for many years has aided greatly in the editing of successive volumes. Professor Hubert S. Loring of Stanford University becomes an associate editor with this present volume. Commencing with Volume XVI, Professor Gordon Mackinney of the University of California will also serve in this capacity. To all three we are deeply grateful: to the first named for his years of generous assistance

IN MEMORIAM

Death came to Carl Louis August Schmidt, professor of biochemistry and chairman of the Division of Biochemistry of the University of California, president of Annual Reviews, Inc., and distinguished scholar, on February 23, 1946, after a lingering and painful illness which he endured with great fortitude.

He was born on March 7, 1885, in Brown County, South Dakota, the only child of Gustav and Friderike Schmidt. When he was about two or three years of age the Schmidt family sold their farm and moved to St. Helena, California. There he received his early schooling and lived until he was of college age. He went through grammar school and high school and was graduated from the St. Helena Union High School in 1904.

As a child he was quite frail, but in later life, though never athletic, he developed a great endurance and capacity for prolonged and exacting work. From his mother he inherited his practical temperament and business ability, and from his father a love of learning and of the great outdoors. His interest in science was kindled by the St. Helena physician, Dr. Osborn, by the scholarly druggist and Civil War veteran, W. H. Smith, and by his various teachers in high school.

When he graduated from high school, his parents sold their St. Helena property and moved to Berkeley to enable him to enter the College of Chemistry of the University of California in the fall term of 1904.

Carl Schmidt's college years were full of variety and interest. In April of 1906, following the San Francisco earthquake, he was sent with his unit of the R.O.T.C. to patrol the crowded Panhandle of the Golden Gate Park for a few days. During his first years in college he worked during the Christmas vacations in the Chemistry Department, stocking lockers and preparing solutions. As early as his junior year he was appointed an assistant in organic chemistry and in his senior year an assistant in physical chemistry. He taught chemistry for one term, giving the laboratory courses, at the A to Zed School and Boone's Academy. These were coaching schools, of which the latter is no longer existent. During his senior year he also assisted the eminent physiologist, Jacques Loeb, then professor of physiology in the University. An event that was to have a profound effect on his life occurred during the year he was assistant in physical chemistry.

Dr. Frederick Cottrell, the regular instructor, was ill and Dr. T. B. Robertson of the Department of Physiology was called upon to give the lectures, while Carl Schmidt was put in charge of the laboratory work.

When he received his B.S. in Chemistry in 1908 he had already published four articles and had been elected to full membership in Sigma Xi.

Two men exerted a profound influence on him during his college years and did much to shape his future career. They were Frederick G. Cottrell, then instructor in physical chemistry, and T. Brailsford Robertson, a man of great intellectual charm and an eminent biochemist of his day. From Cottrell, Carl Schmidt acquired his enthusiasm for accuracy and precision and his interest in physical chemistry and from Robertson his lifelong interest in the chemistry of the proteins. From that time he devoted himself to the study of the physical chemistry of the proteins and of the physical chemical basis of biological processes.

Following graduation he was employed for a little over a year as chemist for the Metropolitan Light and Power Company.

In 1909 he accepted a position in the University with Professor A. E. Taylor to investigate for the Referee Board of the U.S. Department of Agriculture the possible toxicity of the sulfur process and aluminum and copper on foods. In this work he was later joined by Dennis R. Hoagland, now professor of plant nutrition in the University of California. In 1910 he was awarded the M.S. degree in Chemistry on the basis of the work done with Professor Taylor. In 1910, Taylor joined the faculty of the University of Pennsylvania Medical School and took his young assistants with him to Philadelphia to continue the work for the Referee Board.

Carl Schmidt returned to Berkeley in 1912, spent the year in the study of bacteriology, and in 1913 was appointed Chemist, Bacteriologist, and Food Inspector for the City of Berkeley in the Public Health Laboratory, which was but newly established and which he helped equip.

On April 11, 1914, he married Esther May Skolfield, a graduate of Stanford University and bacteriologist at the State Board of Health in Berkeley. For several years they worked and published jointly on problems of bacteriology and immunity.

In 1915 he returned full time to the University of California under a grant from the Hooper Foundation to carry on research in bacte-

riology and immunology. This work on the antigenic properties of proteins, performed under the direction of Professors Gay and Robertson, formed the basis of his thesis for the Ph.D. degree which was awarded in 1916. For the next two years he continued in the University under Professor Gay with the rank of research assistant.

In 1918 he was appointed assistant professor in the newly formed Department of Biochemistry and Pharmacology and served as associate professor from 1920 to 1924. In 1924, following Professor Bloor's departure to the University of Rochester, he was appointed professor and chairman of the Division, a position he held until his death.

Carl Schmidt had supreme faith in the value of research. When he took over as dean the task of reorganizing the College of Pharmacy there was a good deal of skepticism as to what he could do with such a traditionally low-standard field of instruction where research activities were almost nonexistent. The skeptics were soon put to rout. In a few years, he enlisted an able and enthusiastic instructional staff for the College. The quality of research was raised to the level of the rest of the University. This was soon reflected in the quality of the students entering upon the study of pharmacy and in the unprecedented event of awarding the Ph.D. degree in pharmacy.

His two most lasting interests in research were the biochemistry of bile and the chemistry of the amino acids and proteins. In the former he made the important discovery that the bile was a necessary vehicle for absorption of the fat-soluble vitamins. This provided an explanation for the up-to-then unexplained cause of the bleeding tendency in obstructive jaundice: failure of the biliary circulation prevented absorption of vitamin K. His work on the physical chemistry of the amino acids and proteins laid part of the foundation for the important developments in recent years in the clinical use of amino acids and proteins.

For a number of years he worked diligently in the field of immunity. He owed his interest in this subject to F. P. Gay and T. B. Robertson. In these studies he attempted to apply the principles of physical and protein chemistry.

Recognition of his administrative ability brought him many responsibilities inside and outside of the University. In 1937 he was appointed dean and was charged with the responsibility of reorganizing the College of Pharmacy. During the summer of 1938 he served as acting dean of the Medical School in the absence of Dean Langley

Porter. He was on the Advisory Board of the College of Dentistry. He served on numerous University committees and had an important voice in determining University policy. He served as consulting biochemist for the University of California and Southern Pacific Hospitals and as a collaborator of the Western Regional Laboratory of the U.S. Department of Agriculture and of Eli Lilly and Co. He was president of Annual Reviews, Inc., from 1936 on, and served on the editorial committee of the *Annual Review of Biochemistry* from its inception in 1932. At various times he served as secretary and president of the Pacific Coast Section of the Society for Experimental Biology and Medicine and during the last three years was an editor of its *Proceedings*.

He was a member of the editorial committee and of the nominating committee of the American Society of Biological Chemists. He was awarded a fellowship by the Carl Schurz Foundation in 1932 to tour Germany, where he lectured in German at a number of the universities. Research awards were granted him by the Society of the Sigma Xi in 1928 and by the Chemical Foundation, Inc., 1929 to 1934. He was a member of many learned societies and was the moving spirit in organizing the Biochemical Conference which for many years did much to foster informal semiannual meetings of those interested in biochemistry throughout the universities and research institutions of the Pacific Coast.

He was editor of *Chemistry of the Amino Acids and Proteins* and with Frank W. Allen wrote *Fundamentals of Biochemistry*. He published 184 journal articles and supervised the work of twenty-nine students for the Ph.D. degree.

He is survived by his widow, Esther Skolfield Schmidt; three children, Dr. Stanwood S. Schmidt, Alfred C. Schmidt, and Esther F. Schmidt; and one grandson, Alfred Carl Schmidt, Jr.

DAVID M. GREENBERG

ERRATA

Volume XIV, page 170, line 6: *for* Lugg, L. W. H., *read* Lugg, J. W. H.
Volume XIV, page 267, line 27: *for* systems, cysteine, *read* systems, involving cysteine.
Volume XIV, page 271, line 37: *for* nitgrogen-poor, *read* nitrogen-poor.
Volume XIV, page 289, line 38: *for* 0.01 mg. per ml., *read* 0.01 μg. per ml.
Volume XIV, page 294, line 30: *for* adenylpyrophosphate, *read* adenylpyrophosphatase.
Volume XIV, page 369, line 8: *for* indole and glycine, *read* indole and serine.

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BIOLOGICAL OXIDATIONS AND REDUCTIONS

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In spite of continued interruption of the normal research activities of many workers, this has been a productive year in the field of biological oxidations and reductions. Notable advances have been made particularly in three fields, namely, the relation of vitamin B_6 to amino acid decarboxylation and transamination, the interrelation of fatty acid and carbohydrate metabolism, and the study of sulfhydryl groups in enzymes. Several new enzymes have been described and a number of old questions, such as the role of glutathione as a hydrogen transporting agent, have been revived. Limitations of space have not allowed reference to many interesting but isolated observations.

Iron Porphyrin-Containing Enzymes and Carriers

Cytochrome and cytochrome oxidase.—Chaix & Fromageot (1) report that *Propionibacterium pentosaceum* contains cytochromes-*a* and *b*, but not *c*, and a respiratory enzyme which is insensitive to hydrogen sulfide. The respiratory enzyme is inhibited by carbon monoxide but the inhibition is not prevented by illumination.

Greenstein *et al.* (2) have studied the distribution of the cytochrome oxidase system in many normal and neoplastic human and rat tissues. In tissues with a high cytochrome-*c* content (heart, skeletal and diaphragm muscle, liver, kidney, and brain) spontaneous neoplasms are relatively infrequent. Other normal tissues, and benign or early neoplasms usually, contain less cytochrome-*c*. Highly malignant neoplasms in general contain the lowest amounts. In all tissues the cytochrome oxidase system (as measured in suspensions) is limited by the cytochrome-*c* content but the discrepancy between oxidase and cytochrome content is greatest in the malignant tissues.

Kreke & Suter (3) suggest that certain alcoholic yeast extracts, which stimulate the respiration of yeast and tissues, contain autoxidizable substances which substitute for the cytochrome system. The effects of iron inhibitors, cyanide and azide, were antagonized by the extracts but no stimulation could be obtained in the presence of urethanes or amyl alcohol.

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Peroxidase.—The purification and properties of milk peroxidase described by Theorell & Åkeson (4) were reported in a previous review (5). From ultracentrifuge and diffusion studies on recrystallized lactoperoxidase, Theorell & Pedersen (6) calculate the molecular weight to be 92,700. The iron content is 0.070 per cent, indicating one atom of iron per molecule.

More studies on the regeneration of heat-inactivated peroxidase have appeared. Regeneration of peroxidase in briefly heated milk is believed by Fischer (7) to be partly accounted for by regeneration of the protein parts of peroxidase which were reversibly coagulated, and partly by release of enzyme which was adsorbed on the coagulated protein. Regenerated enzyme shows decreased heat resistance which suggests that the effects of heat are not completely reversed. Possibly fitting in with the adsorption and release theory are the observations of Herrlinger & Kiermeier (8) that regeneration of heated horse-radish peroxidase solutions is influenced by the presence of salts and is greater the higher the concentration of the heated enzyme solution; the initial regeneration is greater when the damage to the enzyme protein is increased by prolonged heating at a high temperature.

Bezssonoff & Leroux (9) have found evidence that ascorbic acid and ionized copper or iron constitute a monophenol peroxidase system, while ascorbic acid and copper constitute a polyphenol peroxidase.

Catalase.—Further studies on azide-catalase have confirmed Keilin & Hartree (10) in their belief that catalase action involves reduction of the trivalent hematin iron by hydrogen peroxide and its reoxidation by oxygen. Theorell & Agner (11) had considered this mechanism unlikely but Keilin & Hartree believe that various considerations, particularly the instability of the compound obtained by treating azide-catalase with hydrogen peroxide, invalidate the attempts of Theorell & Agner to determine the valency of its iron by measurements of magnetic susceptibility. Azide, or hydroxylamine, does stabilize reduced catalase considerably (and so acts as an inhibitor), while, with free catalase, reoxidation of the ferrous compound is too rapid for its formation to be observed. Keilin & Hartree show that clearly observable differences in absorption spectrum indicate that hydrogen peroxide reduces the iron in azide-catalase permitting it to form a carbon monoxide derivative which is more stable in air than a free catalase-carbon monoxide derivative. Catalase activity,

in the presence of low concentrations of azide, or of hydroxylamine, is inhibited by carbon monoxide and the inhibition is largely removed by light. The extent of the inhibition depends upon the ratio of carbon monoxide to oxygen, the partition coefficient being of the same order as for cytochrome oxidase. It is pointed out that the change in absorption spectrum of azide-catalase in the presence of hydrogen peroxide provides an extremely sensitive method for detecting the formation of hydrogen peroxide in enzyme catalysed or other oxidations. In alkaline phosphate solution, hydrogen peroxide was shown also to reduce methemoglobin and cytochrome-c.

Kreke, Bartlett & Smalt (12) find that yeast extracts cause a marked increase in the catalase activity of dilute rat liver suspensions. The extract alone shows negligible activity and it inhibits the catalase activity of free hemin. It is believed that the extra activity is due to an iron complex but its mode of action is not yet clear.

OTHER METAL-CONTAINING OXIDASES

Phenol oxidases.—Nelson *et al.* (13, 14) have elucidated the role of tyrosinase as the terminal oxidase in the respiratory chain in potato slices. Their experiments lead them to suggest that this enzyme catalyses the oxidation, by oxygen, of dihydroxyphenylalanine (dopa) to the o-quinone and the latter is reduced again to dopa by the preceding parts of the hydrogen transporting system. l-Tyrosine, present in white potato tubers (*Solanum tuberosum*), is oxidised to dopa by tyrosinase but only in small amount, since the affinity of the enzyme for dopa is so much greater than for tyrosine that small amounts of dopa formed prevent the oxidation of more tyrosine. Tyrosine thus acts as a reserve for the formation of the actual hydrogen carrier, dopa-o-quinone. Any disorganization of the reducing systems, such as bruising the plant and crushing the cells, slows up the reduction of the o-quinone which then undergoes other reactions yielding colored products responsible for the darkening of peeled potatoes. In white potatoes, p-cresol cannot take the place of tyrosine in this system but in sweet potatoes (*Ipomoea batatas* Poir), all the monohydric phenols tried, p-cresol, phenol, and xylenol as well as tyrosine, can do so. The actual hydrogen carrier in sweet potatoes has not been identified.

Tyrosinase, from mushrooms, has been shown by Sizer & Prokesch (15) to catalyse the oxidation of various phenolic toxic principles

of poison ivy and related plants. Oxidation decreases the dermatitis-producing properties of these substances.

Bodine & Hill (16) find that protyrosinase, from grasshopper eggs, does not pass through Whatman No. 42 filter paper but becomes activated by adsorption on the paper. The anionic detergents, sodium dodecyl sulfate and dioctyl sodium sulfosuccinate, produce tyrosinase from protyrosinase and the activation is not reversed by precipitation of the detergents with barium chloride. The enzyme protein complex is apparently fragmented by treatment with dodecyl sulfate and will then pass through Whatman No. 42 paper. Bodine (17) finds that protyrosinase, activated by detergents or heat, acts on both monohydric and dihydric phenols while protyrosinase activated by mercuric chloride or urea shows high monophenolase and low catecholase activity. Thus both animal and plant (18) tyrosinases possess enzymic activities more or less conditioned by chemical treatments.

Some years ago Keilin & Mann (19) purified the enzyme laccase from the latex of *Rhus succedanea,* obtaining a preparation containing 0.24 per cent copper and no iron or manganese. The enzymes from the Indo-Chinese, Burmese, and Japanese lacquer trees were apparently all copper-proteins containing a blue pigment the color of which was not determined by the copper. Bertrand (20) has now obtained a possibly more active preparation from *Rhus succedanea* latex. Studies of the absorption spectrum indicate that the catalyst is not necessarily a protein and spectrographic analysis indicates that managenese is present, while the amount of copper present is at most 0.04 per cent which might have come from reagents used. He also obtained preparations free of the blue color. It may be remembered that many years ago G. Bertrand found manganese in laccase preparations and concluded that this element was concerned in polyphenol oxadise activity. Resolution of the apparent contradiction between the findings of Keilin and Bertrand will be awaited with interest.

Ascorbic acid oxidase.—Lampitt et al. (21, 22) have reopened the controversy concerning the role of protein in ascorbic oxidase activity. They find that a solution containing calcium bicarbonate, an alkali phosphate, and a trace of ionized copper behaves as a synthetic ascorbic oxidase and is inactivated by heating to 55°. They criticise the evidence supporting the view that the oxidase activity of previously isolated copper-protein complexes is exerted by the complex and not by copper ionized from it.

Flavoprotein Enzymes

l-Amino acid oxidase-lactic oxidase.—The preparation and properties of *l*-amino acid oxidase by Green *et al.* (23, 24) was described in a previous review (5). Green and co-workers (25, 26) have now isolated from large quantities of rat kidney a flavoprotein preparation which catalyses the oxidation both of *l*-amino acids and of certain α-hydroxyacids. Though electrophoretic examination indicated a single component, ultracentrifugation showed that the material consists of two components. Both components show the same enzymic activity toward *l*-amino acids and lactic acid but the lighter fraction appears to contain two flavin groups per molecule and the heavy fraction eight; the molecular weights are approximately 138,000 and 552,000. The yellow color and greenish fluorescence of the flavoprotein are bleached by *l*-amino acids or lactic acid and restored on shaking the solution with air. Hydrogen peroxide is formed in either oxidation. The prosthetic group is riboflavin phosphate. It can be split off by heat or acid treatment but is only very slowly dissociated on dialysis. The best preparations still contain a small amount of another colored substance, probably an iron-porphyrin, which appears to be an impurity. The activity of the enzyme is unusually low for a flavoprotein; with *l*-leucine and lactic acid the turnover numbers under optimal conditions are about 6 and 16 molecules per minute respectively.

In connection with the above described enzyme with dual specificity, it is interesting to note that Herner (27) found that mandelic (phenylglycolic) acid is oxidised by kidney and liver tissue and inhibits the oxidative deamination of amino acids. Edlbacher & Grauer (28), from a study of the effects of inhibitors on deamination by guinea pig kidney slices, conclude that there are several different systems which deal with different *l*-amino acids (see also page 23). They also noted a relation between amino acid oxidases and enzyme systems which oxidise hydroxy acids.

Diamine oxidase.—Improved methods for purifying this enzyme from pig kidney have been described by Swedin (29) and Laskowski *et al.* (30). Zeller (31) considers that diamine oxidase is a flavoprotein and Swedin (32) has produced further evidence for this view. Swedin found that the enzyme is very labile, being destroyed below pH 5.9 and at temperatures above 50°C. This possibly explains the failure of attempts by Laskowski *et al.* to split and reunite the enzyme.

Contrary to Zeller, Swedin [see also Laskowski (33)] believes that hydrogen peroxide is not formed as a result of histaminase activity. Nevertheless, he finds that in the presence of peroxidase the rate of oxygen uptake is increased and two atoms of oxygen, instead of one, are taken up.

From a study of the activity of sera from pregnant women, and placenta extracts, toward cadaverine and histamine, Kapeller-Adler (34) has raised doubts concerning the identity of diamine oxidase and histaminase.

Glucose oxidase.—A full account of the previously reported (5, 35) work of Coulthard *et al.* on the isolation, nature, and properties of notatin, the glucose oxidase of *Penicillium notatum,* has now appeared (36). The enzyme is active with xylose and mannose, but far less so than with glucose, and inactive with maltose, arabinose, or galactose. It is probably identical with the well-known glucose oxidase of *Aspergillus niger* and with an enzyme obtained from *P. resticulosum.* The enzyme is a flavoprotein but the nature of the flavin nucleus is not settled. Attempts to reconstitute the enzyme from protein and flavin nucleotides were not successful.

Pyridine Nucleotide Systems

Drabkin & Meyerhof (37) have described a simple method for the determination of dihydrocozymase (DPN · H₂) by iodine titration in neutral solution. The complete ultraviolet absorption spectrum of isolated dihydrocozymase has been studied by Drabkin (38).

Colowick & Price (39, 40) have found that dihydrocozymase, and also guanine, play an essential role in the reactions whereby phosphate is transferred from adenosinetriphosphate to glucose (hexokinase reaction) or to fructose-6-phosphate. The reduced coenzyme thus has an effect which is unconnected with its activity as a hydrogen transporting agent. The authors point out that this effect may be related to the Pasteur effect since the concentration of reduced cozymase would be expected to be much lower under aerobic than under anaerobic conditions.

Isocitric dehydrogenase.—Ochoa (41) has analysed the mechanisms concerned in the biological oxidation of isocitric acid to α-ketoglutaric acid and carbon dioxide. A dialysed extract from pig heart was found to contain enzymes which catalyse the following two reversible reactions.

(*a*) Isocitric acid + triphosphopyridine nucleotide (TPN) ⇌ oxalo-succinic acid + dihydro-triphosphopyridine nucleotide (TPN · H$_2$)

(*b*) Oxalosuccinic acid ⇌ α-ketoglutaric acid + CO$_2$

Reaction (*a*) is catalysed by isocitric dehydrogenase and reaction (*b*) is catalysed by a newly defined enzyme, oxalosuccinic carboxylase, which requires manganese ion for its activity. Summation of the two reactions gives:

(*c*) Isocitric acid + TPN ⇌ α-ketoglutaric acid + CO$_2$ + TPN · H$_2$

The coenzyme, reduced either by reaction (*a*) or by glucose-6-phosphate and *Zwischenferment*, can be reoxidised by the heart enzyme with α-ketoglutarate plus carbon dioxide in the presence of manganese. The net result of reversal of reaction (*c*) by combination with the hexosemonophosphate dehydrogenase system would be the dismutation:

(*d*) CO$_2$ + α-ketoglutaric acid + glucose-6-phosphate ⇌ isocitric acid + 6-phosphogluconate

If aconitase were present, over 90 per cent of the isocitric acid would be removed to give *cis*-aconitate and citrate, and reaction (*d*) might be shifted well to the right with consequent notable carbon dioxide fixation.

The equilibrium constants of reactions (*a*) and (*c*) were experimentally determined and that of reaction (*b*) deduced.

Ochoa (42) has also shown that the isocitric dehydrogenase system can be linked with the cytochrome system by cytochrome reductase. Cytochrome-*c* was reduced in the presence of a purified preparation containing isocitric dehydrogenase and oxalosuccinic carboxylase when isocitrate, manganese salt, TPN, and cytochrome reductase were added.

Moulder, Vennesland & Evans (43) describe a dismutation which occurs in pigeon liver and muscle extracts supplemented with manganese ions and either di- or triphosphopyridine nucleotide (DPN or TPN), whereby isocitrate and pyruvate react to give oxalosuccinate and lactate. In liver extract the oxalosuccinate is then decarboxylated yielding α-ketoglutarate; with muscle extract a decarboxylase preparation must be added. Isocitric dehydrogenase from other sources has always been found specific for TPN, and lactic and malic dehydrogenases for DPN. But these authors find that the isocitric,

lactic, and malic dehydrogenases of pigeon liver and muscle are active with both DPN and TPN.

Triosephosphate dehydrogenase-triose dehydrogenase.—Cori *et al.* (44) have described a simple method for isolating *d*-glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle extract. They obtained a crystalline protein which is homogeneous in the Tiselius apparatus. The yield was equivalent to 7 per cent of the extracted protein. The activity is of the same order as that of the yeast enzyme of Warburg & Christian (45). A reducing agent is necessary for the full activity of the muscle enzyme. Caputto & Dixon (46) obtained triose dehydrogenase in crystalline form and discovered that it was about 300 times as active toward glyceraldehyde phosphate as toward glyceraldehyde. They concluded that triose and triosephosphate dehydrogenases are identical. The presence of inorganic phosphate is necessary for both activities.

In a spectrophotometric study of the kinetics of the oxidation and phosphorylation of *d*-glyceraldehyde-3-phosphate to *d*-glyceric acid-1,3-diphosphate, in the presence of cozymase and dehydrogenase from yeast, Drabkin & Meyerhof (37) have obtained evidence that an unstable addition product is formed as a precursor of glyceric acid diphosphate. The dimeric glyceraldehyde phosphate, synthesized by Baer & Fischer (47), is not concerned in the reaction. Inorganic phosphate appears to have another function in the equilibrium besides the final formation of the glyceric acid diphosphate or the formation of a diphosphate precursor.

OTHER OXIDASE AND DEHYDROGENASE SYSTEMS

Pyruvic oxidase.—Stumpf (48) has prepared from disintegrated *Proteus vulgaris* an enzyme, associated with macro particles, which specifically catalyses the oxidation of pyruvate to acetate and carbon dioxide. For maximum activity, the enzyme requires the addition of diphosphothiamine and one of a number of bivalent cations, manganese being the most active, though it is believed that magnesium is the metal in the natural system. It seems probable that the diphosphothiamine group is not very dissociable from the protein but that a phosphatase splits some of the diphosphothiamine during the preparation. The metal is bound to the protein but can be split off at pH 4.0. The enzyme from *Proteus vulgaris* and a similarly prepared enzyme from *Escherichia coli* do not form acetyl phosphate when

phosphate is present nor do they require inorganic phosphate for their activity. In the latter respect these enzymes differ from another enzyme system from *E. coli* which catalyses the formation of acetyl phosphate and formate from pyruvate (49, 50), and from several other bacterial enzymes, which cause pyruvate to undergo various types of reaction.

Lipoxidase.—With a considerably purified preparation of soybean lipoxidase, Cosby & Sumner (51) have found no evidence that the enzyme requires an activator or can be split into apoenzyme and coenzyme fractions. Balls *et al.* (52) had previously found that the presence of a polypeptide-like substance from heated soybean meal extract, which behaves rather as a protective agent than as a coenzyme, is necessary for the full activity of purified enzyme.

Holman & Burr (53) have found that linoleic and linolenic acids and their esters and methyl arachidonate are converted by lipoxidase into substances, presumably conjugated unsaturated carbonyl compounds, which show increased absorption at 2300 and 2700 Å. The increased absorption produced by the action of the enzyme is similar to that observed in the autoxidation of fats and fatty acids, suggesting that the same products are produced in both cases. Oleic acid and its ester, and the transisomers of linoleic and linolenic acids, are not attacked by the oxidase and their absorption spectra are not altered.

Uricase.—In the oxidation of uric acid under the influence of uricase, Felix *et al.* (54) and Schuler (55) found that the ratio of carbon dioxide produced to oxygen consumed varied with the pH, and they believed that the uric acid was oxidised to hydroxyacetylene-diureine-carboxylic acid which was then decarboxylated under the influence of another catalyst to yield allantoin. Klemperer (56) has now made a detailed study of the oxidation of uric acid with beef kidney and pig liver uricase and has produced evidence that the primary product is an unstable compound which decomposes, probably spontaneously, into three reaction products: allantoin (and carbon dioxide), uroxanic acid, and hydroxyacetylene-diureine-carboxylic acid. The relative amounts of these products formed depends upon the pH and the nature of the buffer used.

Oxaloacetic oxidase.—An enzyme which catalyses the oxidation of oxaloacetic acid by molecular oxygen has been found in various tissues by Vennesland & Evans (57). With extracts of ground pig heart it was shown that dialysis leads to inactivation and activity can

be restored by the addition of manganese chloride. Malonic acid was identified as the oxidation product and manometric measurements indicated that the reaction proceeds as follows:

$$COOH \cdot CH_2 \cdot CO \cdot COOH + \tfrac{1}{2}O_2 \rightarrow COOH \cdot CH_2 \cdot COOH + CO_2.$$

Malonic acid has been used by many workers as an enzyme inhibitor, particularly of succinic dehydrogenase, in studies of the Krebs and Szent-Györgyi cycles. That this substance can actually be formed in tissues from one of the members of these cycles is suggestive.

Glutathione oxidase.—Interest in glutathione as a possible intermediary hydrogen transporting agent in tissue respiration has been revived by Ames & Elvehjem (58). These authors find, in mouse kidney extract, a system which causes rapid oxidation of reduced glutathione (GSH) especially when cytochrome-c is added. In the presence of optimal concentrations of glutathione and cytochrome-c, a Q_{o_2} of 418 (corrected for autoxidation) was obtained, based on the dry weight of fresh tissue represented by the extract. The authors state that reduction of cytochrome-c by GSH is much more rapid in the presence of tissue (reoxidation being inhibited by cyanide) than without tissue. This suggests the activity of a glutathione dehydrogenase. The system is heat-labile and it is inhibited by low concentrations of diethyldithiocarbonate, as well as by cyanide, which suggests that a copper-protein is concerned.

The oxidation of the "fixed-SH" of proteins by oxidised glutathione (GSSG) is well known and certain dehydrogenase systems have been shown to reduce GSSG. Ames & Elvehjem therefore suggest that glutathione serves as a "coenzyme" in tissue oxidations through the cytochrome system. In earlier work, Hopkins & Elliott (59) had shown that liver from well-fed animals contains metabolites which reduce GSSG, and that the GSH formed could be reoxidised in the tissue, though the rate of oxidation did not correspond to more than a small part of the total respiration. Though the reoxidation was inhibited by cyanide, they did not believe that the cytochrome system was involved because carbon monoxide and heating to 70° did not inhibit the oxidation. However, liver is very rich in the cytochrome system and it is possible that a sufficient amount remained active to catalyse the oxidation of GSH at the usual rate, this rate being limited by other factors.

Amine oxidases.—Bernheim & Bernheim (60) have found that heart, artery, and intestinal muscle of various animals contain an en-

zyme system which destroys tyramine by oxidation of both the amine and hydroxy groups. The system differs from the well-known amine oxidase, present in liver, kidney, and other tissues, which attacks only the amine group.

Blaschko (61) has presented evidence indicating that the mescaline oxidase of rabbit liver is a different enzyme from amine oxidase.

Inorganic substances.—Lang (62) discovered in animal tissues an enzyme called rhodanese which catalyses the formation of thiocyanate (rhodanate) from hydrocyanic acid and thiosulfate, or colloidal sulfur, according to the reaction: $HCN + Na_2S_2O_3 = NaSCN + NaHSO_3$. This enzyme has now been isolated from beef liver and considerably purified by Cosby & Sumner (63).

The ability of an organism to produce an adaptive catalytic system without growth has been demonstrated by Knox & Pollock (64) in the case of the reduction of sodium tetrathionate to thiosulfate by *Bact. paratyphosum* B.

Surface catalysts and the production of virus.—Yamafuji *et al.* (65, 66) report that dried tissue preparations possess "surfaces" which activate oxygen in such a way that it reacts much more rapidly in various enzymic oxidation systems. Under certain circumstances tissue catalase activity may be diminished *in vivo*. Hydrogen peroxide formed in reactions catalysed by the "surface" activators may then cause the production of viruses by denaturation or polymerization of nucleoprotein (67, 68, 69). Confirmation from other laboratories of these striking reports would be most interesting.

STUDIES ON INHIBITORS

A useful review of the various types of enzyme inhibitors and their significance has been published by Singer (70). An extensive review and account of experimental work on fluoride inhibition has been published by Borei (71). Fluoride attacks the protein part of cytochrome-*c*. Paschkis *et al.* (72, 73) find that thiouracil and sulfonamides, which are known to suppress formation of thyroid hormone, in low concentrations inhibit the cytochrome oxidase (*p*-phenylenediamine oxidation) of thyroid tissue considerably but do not affect that of bone marrow or kidney.

A marked decrease in the cytochrome-*c* concentration and in the activity of the cytochrome, succinic, pyruvic, and glutamic oxidase systems has been found in the kidneys of experimental hypertensive dogs by Raska (74, 75). A renin preparation, and preparations from

kidneys of hypertensive dogs, inhibited the same systems and the amine oxidase. An increase in the concentrations of respiratory enzymes and coenzymes occurred in hypertrophic kidney.

Michaelis & Thatcher (76) find that the antibiotic, citrinin, inhibits the oxidation of lactate and glucose by *S. aureus,* an organism which is susceptible to citrinin, but scarcely affects these oxidations in the resistant *B. coli.* Hurst (77) has discussed the mode of action of insecticides and drugs in relation to phenolase, peroxidase, and other systems and to the structural components of insect cuticle and tissue.

Greig & De Turk (78) have obtained and purified from liver a thermolabile material resembling a globulin which inhibits the *d*-amino acid oxidase. The effect is more marked at relatively low pH and seems to be reversible. Kinetic studies indicate that the inhibitor does not compete with either the flavin dinucleotide or the substrate but it probably forms an apoenzyme-coenzyme-substrate-inhibitor complex. The results of Cheney (79) indicate that caffeine, like cyanide, inhibits that fraction of the respiratory activity of *Arbacia* eggs which is concerned with activity, that is, cell division.

Sulfhydryl groups.—Of particular interest is the number of enzymes in which a sulfhydryl group has been shown, by inhibitor studies, to be essential for activity. Barron & Singer (80) have surveyed a large number of enzymes concerned with carbohydrate, fat, and protein metabolism, using particularly *p*-chloromercuribenzoate, certain trivalent organic arsenicals, and iodoacetamide as agents attacking sulfhydryl groups. They discuss the differences between various reagents that act upon sulfhydryl groups and the variations in lability of different enzyme sulfhydryl groups. In most cases, complete or partial reactivation of the enzymes by reduced glutathione provided further proof of the dependence of the enzymes upon sulfhydryl groups. The authors consider that the main function of glutathione is the continuous reactivation of sulfhydryl enzymes which have been inactivated by oxidising agents in the cell.

The following have been shown to be sulfhydryl enzymes by Barron & Singer and other workers. [References are given only to very recent papers; for others see (70, 80).] :

Systems responsible for pyruvate oxidation, dismutation, decarboxylation, and acetoacetate and acetylmethyl carbinol formation; hexosemonophosphate, phosphoglyceraldehyde, glycerol, α-ketoglutaric, succinic, malic, and alcohol (yeast) dehydrogenases; synthesis of α-ketoglutarate; adenosinetriphosphatase; phospho-
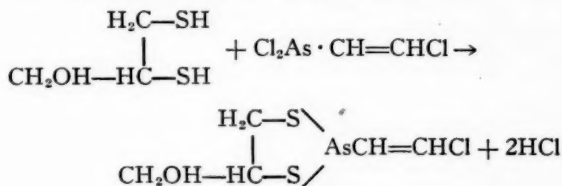
glucomutase; phosphorylase; hexokinase; pancreatic amylase; β-amylase (barley, 81); stearate (bacteria and liver), oleate (bacteria), and acetate (yeast, bacteria) oxidases; β-hydroxybutyric dehydrogenase; pancreatic lipase and esterase; cerebrosidase; *d*-amino acid oxidase; mono-amine oxidase; *l*-glutamic dehydrogenase; transaminase (both aspartic and alanine); urease; cathepsins; cathepsin dipeptidase; papain; bromelin; hemolysins; lysozyme; choline oxidase; cholinesterase; and choline acetylase (82, 83).

The activity of the following enzymes does not appear to depend upon free sulfhydryl groups:

Cytochrome oxidase; catalase; heart flavoprotein; alcohol (liver), lactic and isocitric dehydrogenases; uricase; diamine oxidase; polyphenol oxidase; oleic oxidase (peanuts); carbonic anhydrase; acid phosphatase; arginase; pepsin; and trypsin.

Extremely interesting results of this type of study have recently been disclosed and reviewed by Peters, Stocken & Thompson (84) and Waters & Stock (85). It appears that chlorarsines such as lewisite ($ClCH : CH \cdot AsCl_2$) penetrate the keratin layer of skin by virtue of their lipoid solubility and are rapidly hydrolysed to give arsenoxides which combine with protein sulfhydryl groups. The pyruvic oxidase system of brain is particularly sensitive to inhibitors of sulfhydryl enzymes and proved a useful test system in these studies. Monothiol compounds are ineffective in counteracting the action of lewisite or arsenite and, in fact, thiol arsenical compounds are as toxic as the arsenicals themselves, presumably because they dissociate readily. But the dithiol compound 2,3-mercartopropanol or BAL (British anti-lewisite) is highly effective since it combines with arsenious compounds to give a stable cyclic structure:

$$
\begin{array}{c}
H_2C\text{---SH} \\
| \qquad\qquad + Cl_2As \cdot CH{=}CHCl \rightarrow \\
CH_2OH\text{---HC---SH}
\end{array}
$$

$$
\begin{array}{c}
H_2C\text{---S}\diagdown \\
| \qquad\qquad AsCH{=}CHCl + 2HCl \\
CH_2OH\text{---HC---S}\diagup
\end{array}
$$

There is evidence that lewisite and other trivalent arsenicals combine with neighboring sulfhydryl groups in proteins to give stable

arsenical rings, but BAL can compete successfully with protein sulf-
hydryl groups for the arsenic. It protects against and even reverses
the effect of lewsite on pyruvic oxidase and other enzymes. It pro-
tects against the effects of lewisite and other arsenicals on the skin
and eye *in vivo* and on trypanosomes, spermatozoa, and tissue cul-
tures, and even reverses effects if applied soon enough. Systemically
it aids in the urinary excretion of arsenic. BAL is a strong reducing
agent and reduces methemoglobin to hemoglobin and maintains cyto-
chrome-c in the reduced state. It inhibits brain glycolysis, presum-
ably by combination with metal-protein components of the glycolytic
mechanism, but it reactivates succinoxidase which has been inhibited
by heavy metal. Oxidised BAL is itself an inhibitor of sulfhydryl
enzymes.

Colwell & McCall (86) have suggested that the antibacterial action
of 2-methyl-1,4-napthoquinone is due to blocking of essential sulfhy-
dryl groups. The same mechanism has been suggested (87, 88) for
the action of penicillin and other antibiotics. Lyons (89) surveys evi-
dence which indicates that the production of fibrin involves the re-
lease of sulfhydryl groups in fibrinogen followed by the formation of
—SS— bridges as a result of oxidation by 2-methyl-1,4-napthoqui-
none or by a component of thrombin which contains vitamin K or
other quinone structures.

Oxygen poisoning.—Effects of high pressures of oxygen on tissue
metabolism are referred to later. Stadie *et al.* (90, 91) have surveyed
the effects *in vitro* on a number of enzyme systems. Under high oxy-
gen pressure, succinic dehydrogenase is slowly inactivated. Xanthine
oxidase is very sensitive to oxygen. d-Amino acid oxidase is very
susceptible to air or oxygen in cytolysed tissue suspension but is not
affected by high oxygen tension in slices. Cell-free preparations of
choline acetylase are rapidly destroyed by oxygen but acetylcholine
synthesis by brain slices or suspensions is not affected. High pressures
of oxygen are found to exercise no effect on cytochrome oxidase,
cytochrome-c, uricase, catalase, pepsin, cholinesterase, or carbonic
anhydrase.

The effect on succinic dehydrogenase appears to be direct and
not mediated by the cytochrome system. It probably affects the sulfhy-
dryl groups since the enzyme can be reactivated by glutathione or
cysteine or protected by malonate or high concentrations of succinate.
However not all sulfhydryl enzymes are susceptible to oxygen poison-
ing since cholinesterase is not affected.

REACTIONS CONCERNED IN METABOLIC CYCLES

For reviews on pyruvate and fatty acid metabolism which cover the background of the following sections, see Stotz (92) and Stadie (93).

Oxidation of fatty acid derivatives.—In the review by Lardy & Elvehjem (94) a diagram was presented which synthesized current views concerning the pathways of oxidative catabolism of fat and carbohydrate. Further evidence has appeared which indicates that a two-carbon fragment is concerned in both fat and carbohydrate oxidation and that this fragment enters the tricarboxylic acid cycle.

As described in a previous review (5), Breusch (95, 96) and Wieland & Rosenthal (97) found that citric acid was produced from oxaloacetic acid plus pyruvic, or more actively, acetoacetic acid, with kidney, heart, and brain tissues or extracts. Breusch found that a number of β-ketonic acids could replace acetoacetic acid. He therefore proposed a theory whereby a fatty acid is oxidised to the β-keto acid which reacts with oxaloacetic acid to yield citric acid and the fatty acid with chain shortened by two carbon atoms. The citric acid is oxidised via the tricarboxylic acid cycle to oxaloacetic acid again. By repetition of this mechanism the fatty acid chain is continuously shortened by two carbons at a time. These reports have raised some controversy and have stimulated some interesting work.

Krebs & Eggleston (98, 99) found that the removal of acetoacetate by minced heart and kidney was accelerated by the presence of oxaloacetate. But the acetoacetate was quantitatively converted to β-hydroxybutyrate by dismutations with malate and with α-ketoglutarate formed from oxaloacetate. The dismutation with malate apparently maintained a greater supply of oxaloacetate which could react with pyruvate to give an increased yield of citrate.

Other evidence strongly supports the belief that fatty acid derivatives are oxidised via the tricarboxylic acid cycle. Hunter & Leloir (100) have prepared washed kidney cortex particles which produce citrate from acetoacetate and oxaloacetic acid. In this case, β-hydroxybutyrate formation could account for only a small part of the acetoacetate disappearance. Two molecules of citrate appeared to be formed for each molecule of acetoacetate which disappeared, which is in agreement with the view of Wieland & Rosenthal. The latter authors had found that the presence of oxygen was necessary for the condensation to occur in kidney brei even though no oxidation appeared to

be involved. Hunter & Leloir find that simultaneous oxidation of α-ketoglutarate seems necessary for the condensation. For the rapid removal of acetoacetate and formation of citrate, α-ketoglutarate (or glutamate or glutathione from which α-ketoglutarate can be formed) as well as oxaloacetate had to be added to the suspensions. But the oxidation of the α-ketoglutarate could occur aerobically at the expense of oxygen or anaerobically at the expense of reduction of excess oxaloacetate. It is probable (see below) that the actual condensation product is isocitrate or *cis*-aconitate which is largely converted to citrate by aconitase. The explanation of the differences between these findings and those of Krebs & Eggleston, and the mechanism by which the condensation is linked to α-ketoglutarate oxidation remain to be elucidated. Hunter & Leloir suggest that the oxidation of α-ketoglutarate is coupled with a change in acetoacetate, possibly the formation of an unstable two-carbon compound which precedes condensation with oxaloacetate.

Lehninger (101, 102) has prepared rat liver suspensions which in the presence of adenosinetriphosphate oxidise fatty acids. Octanoate yields two molecules of acetoacetate. When the Krebs cycle is inhibited by malonate the suspensions oxidise pyruvate, two molecules, to give one molecule of acetoacetate and two of carbon dioxide. If fumarate and malonate are added, the yield of acetoacetate from either octanoate or pyruvate is greatly diminished and citrate accumulates. Acetoacetate itself forms no citrate in the presence of fumarate. It is concluded that reactive fragments, probably two-carbon, which are produced on oxidation of either pyruvate or fatty acid may react together, to give acetoacetate, or with oxaloacetate (from fumarate) to give citrate. None of a series of known four-carbon or two-carbon compounds including acetate, acetylphosphate, and acetylpyrophosphate was found to be involved in the formation of acetoacetate. The nature of the reactive two-carbon fragment is still unknown.

Buchanan *et al.* (103) have demonstrated, by isotope studies, that acetoacetic and acetic acids can be oxidatively metabolized by way of the tricarboxylic acid cycle. The aerobic disappearance of acetoacetate in guinea pig kidney suspension was stimulated threefold by addition of any acid of the tricarboxylic acid cycle. When acetoacetate or acetate, labeled with isotopic carbon in the carboxyl and carbonyl positions, was used, it was found that isotopic carbon became incorporated in α-ketoglutarate, succinate, and fumarate, and that this

was not due to evolution and refixation of isotopic carbon dioxide. From the fact that the labeled carbon was found almost exclusively in the γ-carboxyl group of α-ketoglutarate, it was concluded that citrate could not be a significant intermediate. But the results could be well accounted for if a condensation occurred between acetic acid, or a two-carbon compound derived from acetoacetic acid, and oxaloacetate to give *cis*-aconitic, or isocitric, acid which is then oxidatively degraded to oxaloacetic acid via the Krebs cycle. Rittenberg & Bloch (104) report evidence that acetic acid enters the tricarboxylic acid cycle *in vivo*. The intermediates of the cycle cannot be isolated from the intact animal but glutamic and aspartic acids which are in biological equilibrium with ketoglutarate and oxaloacetate can be isolated from protein. When acetate labeled with C^{13} was fed to rats and mice, the dicarboxylic amino acids, especially of the liver, were found to contain C^{13} in amounts greater than could be accounted for by carbon dioxide fixation.

Since carbohydrate can be synthesised from the compounds of the cycle it would be expected that integration of fatty acid metabolism into the cycle would provide a means whereby fatty acid carbon may be incorporated in carbohydrate. Lorber, Lifson & Wood (105) have shown that labeled acetate carbon can be incorporated into liver glycogen *in vivo* by a pathway not involving carbon dioxide liberation and refixation.

Weinhouse, Medes & Floyd (106) have shown by isotope studies that slices of liver and also of kidney actively cause the condensation of acetate to acetoacetate. Oxidative energy is required and the evidence suggests that acetic acid condenses with an active two-carbon molecule produced either from the acetic acid or from other metabolites in the tissue. From consideration of the isotope content of the respiratory carbon dioxide and of the acetoacetate, Weinhouse *et al.* consider it possible that acetate is converted to acetoacetate before undergoing oxidation. This would suggest that the active two-carbon compound which enters the tricarboxylic acid cycle may not be formed directly from free acetic acid.

Carbon dioxide fixation in heterotrophic organisms.—While the fixation of carbon dioxide by reversal of the decarboxylation of oxaloacetate has been demonstrated with bacterial preparations (107, 108), some difficulty has been experienced in proving the mechanism of the initial fixation reactions in animal tissue. Evans *et al.* (43, 109) found that when pyruvate and fumarate are incubated with pigeon liver ex-

tracts, supplemented with manganese and di- or triphosphopyridine nucleotide, a series of reactions occurs whereby lactate and carbon dioxide are produced while the pyruvate concentration remains unchanged: fumarate is changed reversibly to malate, the malate reacts reversibly with pyruvate to give oxaloacetate and lactate, and the oxaloacetate breaks down to pyruvate and carbon dioxide. In the course of the reaction a rapid fixation of labeled carbon dioxide occurs. Wood, Vennesland & Evans (110) have shown that the fixed carbon appears exclusively in the carboxyl groups of the lactate, pyruvate, malate, and fumarate concerned in the reactions. While this is compatible with the belief that the carbon dioxide is fixed by reversal of the decarboxylation of oxaloacetate, they were unable to demonstrate that this reaction occurs. However, Utter & Wood (111) have now shown that this reaction occurs readily with pigeon liver extract when adenosinetriphosphate, as well as manganese, is added. As expected, the labeled carbon of the $C^{13}O_2$ used appears in the carboxyl group adjacent to the methylene group. The role of the ATP is not yet clear.

Until very recently pigeon liver was the only vertebrate tissue from which cell-free extracts capable of converting carbon dioxide into organic combination have been prepared. As was described on a previous page Ochoa (41) has now prepared an extract from pig heart which fixes carbon dioxide by the reversal of the decarboxylation of oxalosuccinate to α-ketoglutarate.

We thus have proof of carbon dioxide fixation by $C_3 + C_1$ and $C_5 + C_1$ reactions in animal tissue. In bacteria the $C_3 + C_1$ reaction was previously proven (107, 108). Lipmann & Tuttle (112) and Utter, Lipmann & Werkman (113, see also 94) have further demonstrated a $C_2 + C_1$ reaction, namely, the reversal of the phosphoroclastic splitting of pyruvic acid to acetyl phosphate and formic acid in extracts of *Clostridium butylicum* and *E. coli*. Since formic acid is in equilibrium with carbon dioxide and hydrogen in the cells, the reaction can involve a fixation of carbon dioxide. It was shown that the intact cells of *E. coli* acting upon pyruvic acid in the presence of $NaHC^{13}O_3$ fix C^{13} in the carboxyl group of pyruvic acid.

Photosynthesis.—Franck, Pringsheim & Ladd (114) have studied the phosphosynthetic oxygen production of the algae *Scenedesmus* and *Chlorella* under extreme anaerobic conditions. Time curves of oxygen production, and light saturation curves, during continuous illumination and following single flashes, indicate that anaerobic me-

tabolism gives rise to the production of a substance which inhibits the oxygen-liberating system and that another product has a less specific effect, resembling that of a narcotic, on cell metabolism. Deprivation of carbon dioxide, or addition of cyanide which inhibits the catalyst primarily responsible for the fixation of carbon dioxide, has little effect on the evolution of oxygen at first but greatly diminishes the steady rate of oxygen production. The observations and conclusions are in agreement with the theories of Gaffron (115) and Franck & Herzfeld (116).

In the light of his work on the reversibility of the phosphoroclastic splitting of pyruvate, Lipmann (112) has suggested that a continuing series of such reactions may be involved in photosynthesis. A carboxyl group is phosphorylated and then undergoes reductive carboxylation yielding the keto acid. This is hydrogenated to give the hydroxy acid; the carboxyl group of this is then phosphorylated and the cycle is repeated.

Decarboxylation.—Ochoa & Weisz-Tabori (117) have prepared from pig heart an enzyme which catalyses the decomposition of oxalosuccinate (the first oxidation product of isocitrate) to α-ketoglutarate and carbon dioxide. Indications of the presence of a similar enzyme in pigeon liver were also obtained by Moulder *et al.* (43). The presence of manganese ions is necessary while magnesium ions have almost no effect. In this respect the enzyme resembles the oxaloacetate carboxylase from liver (109) and the pyruvic carboxylase from *E. coli* (108). It differs from the oxaloacetic and pyruvic enzymes of *Micrococcus lysodeikticus* (107, 118), from the pyruvic enzyme from yeast (119, 120), and from the preparation from heart which attacks α-ketoglutarate, α-ketobutyrate, or pyruvate (121). All of these enzymes require or contain magnesium, and all except the oxaloacetic enzyme involve diphosphothiamine.

Six specific bacterial amino acid decarboxylases have been obtained in cell-free preparation, several of them have been considerably purified, and their properties have been recorded by Gale, Epps & Taylor. $l(+)$-Lysine decarboxylase was purified from *E. coli* and *B. cadaveris* (122), $l(-)$-tyrosine decarboxylase from *Strep. fecalis* (123), $l(-)$-histidine decarboxylase from *Cl. welchii* (124), $l(+)$-arginine decarboxylase from *E. coli*, $l(+)$-ornithine decarboxylase from *Cl. septicum*, and $l(+)$-glutamic acid decarboxylase from *Cl. welchii* (125). The specificity of these enzymes seems to be complete except that hydroxy derivatives of lysine and tyrosine are also

attacked. In all cases the corresponding amine and carbon dioxide were identified as reaction products. The enzymes specific for tyrosine and ornithine apparently dissociate spontaneously, and those specific for lysine and arginine can be resolved into apoenzyme and coenzyme, and can be reconstituted. The coenzyme is common to all four decarboxylases. Gale & Epps (126) found that the codecarboxylase is widely distributed in animal and plant tissues, yeast, and bacteria, and they obtained a highly purified preparation from yeast. Epps (124) and Taylor & Gale (125) could not resolve the enzymes specific for histidine and glutamic acid nor find evidence that they contained codecarboxylase. However, Umbreit *et al.* (127, 128) have resolved glutamic decarboxylase, purified from *E. coli,* as well as arginine and tyrosine decarboxylases, and also have shown that the same coenzyme is active with the different decarboxylases.

Umbreit, Bellamy & Gunsalus (128, 129, 130) have concluded that the natural coenzyme isolated by Gale & Epps is a phosphorylated pyridoxine derivative. With apoenzyme preparations obtained from *Strep. fecalis* grown in vitamin B_6-deficient medium, or with apoenzyme otherwise prepared, they were able to activate tyrosine decarboxylase by the addition of pyridoxal and adenosinetriphosphate or by a synthetic substance obtained on treating pyridoxal with phosphorylating agents. Bellamy *et al.* (131) have found that pyridoxine, pyridoxamine, and pyridoxal are all converted into codecarboxylase by organisms capable of using them as sources of vitamin B_6. Cell suspensions or preparations from *Strep. fecalis* R are less efficient in forming codecarboxylase than are growing cells, and pyridoxamine is converted only in the presence of keto acid (pyruvic). This suggests that pyridoxamine is first converted to pyridoxal by a transamination reaction. Though Gale & Epps reported that their purified codecarboxylase contained no phosphorus, Baddiley & Gale (132) find that it actually contains 0.8 to 1.0 per cent phosphorus. They find that excess of pyridoxal phosphate gives the same activation of the tyrosine, lysine, arginine, and ornithine decarboxylases as does excess of natural codecarboxylase.

Gale (133) has applied the specific bacterial decarboxylases to the determination of amino acids in protein hydrolysates.

The distribution and nature of amino acid decarboxylases of mammalian tissues has been recently reviewed by Blaschko (134). In general the activity of these enzymes in animal tissues is low. While, as noted above, the same bacterial enzyme decarboxylates both ty-

rosine and dihydroxyphenylalanine (dopa), this is not true of the animal decarboxylases. Page (135) has studied the preparation, kinetics, properties, and distribution of dopa decarboxylase in tissue extracts, using a biological method (effect on blood pressure) for the estimation of the hydroxytyramine formed in the reaction. The enzyme was found to be very active in guinea pig kidney, less so in kidneys of rabbits and monkeys, still less in human kidneys, and absent from all rat tissues and from human placenta. Green, Leloir & Nocito (136) have effected considerable purification of dopa decarboxylase from pig kidney. When a solution of the purified enzyme was dialysed against dilute ammonia it became virtually inactive but could be partly reactivated by addition of the yeast codecarboxylase of Gale & Epps or by the phosphorylated pyridoxal of Umbreit *et. al.*, together with a reducing substance such as cysteine or glutathione.

Transamination.—Braunstein & Kritsman (137), who discovered transamination, believed there were two "aminopherases" in animal tissues specific for glutamic and aspartic acids (or α-ketoglutaric and oxaloacetic acids) respectively but quite unspecific for the α-keto acid (or amino acid) partner in the reactions. Cohen (138), however, using more specific methods found that transamination reactions are much more limited. He believed that there is a single enzyme, transaminase, which catalyses rapidly only the following two reactions:

(*a*) Aspartate + α-ketoglutarate ⇌ oxaloacetate + glutamate

(*b*) Alanine + α-ketoglutarate ⇌ pyruvate + glutamate

Green, Leloir & Nocito (136) have separated, and obtained in highly purified condition, two transaminases from pig heart. The enzymes, which they call aspartic-glutamic transaminase and alanine-glutamic transaminase, catalyse the above reactions (*a*) and (*b*) respectively. The aspartic enzyme was obtained in two active forms which differed in electrophoretic mobility but behaved alike in the ultracentrifuge. Elegant methods were applied to the determination of the kinetics of the two reactions. Turnover numbers of 640 and 8600 per minute were estimated for the aspartic and alanine enzymes respectively. It appears that the aspartic enzyme might account for 1.6 per cent of the dry weight of the heart muscle, the alanine enzyme for 0.12 per cent. The enzymes are quite specific for the above reactions except that with the aspartic enzyme cysteic acid may be substituted for aspartic and mesoxalic acid for oxaloacetic, though the activity is then much smaller. Similarly some activity occurs with

the alanine enzyme when α-aminobutyric acid is substituted for alanine or when mesoxalic or α-ketobutyric acid is substituted for pyruvic. Moulder, Vennesland & Evans (43) find that pigeon liver extracts apparently cause transamination between aspartate and pyruvate. The oxaloacetate formed is decarboxylated (manganese being added) to pyruvic acid, so that the net reaction is a conversion of aspartate to alanine and carbon dioxide. Green *et al.* believe that such a transamination does not occur directly but is the summation of the activities of his two enzymes mediated by catalytic amounts of α-ketoglutarate-glutamate. On the other hand, Cohen (138) was not able to bring about this transamination even with added α-ketoglutarate.

Schlenk & Snell (139) found that transaminase preparations from pig heart contained vitamin B_6, and that tissues from vitamin B_6-deficient rats showed decreased transaminase activity. Addition of pyridoxal and adenosinetriphosphate usually increased the activity of liver suspensions from deficient animals. Schlenk & Fisher (140) obtained a highly purified preparation of aspartic-glutamic transaminase from pig heart and found that it contained 500 γ of firmly bound pyridoxine derivative per gram. Though it was not shown that a prosthetic group could be reversibly dissociated from their purified transaminases, Green *et al.* (136) have provided ample evidence that both enzymes contain a derivative of pyridoxal which is identical with codecarboxylase. Heated transaminase could replace the codecarboxylase of Gale & Epps or the phosphorylated pyridoxal of Umbreit *et al.* as coenzyme for dopa carboxylase, the coenzyme activity being fairly well correlated with the original transaminase activity. The substance from heated transaminases, natural codecarboxylase, and pyridoxal phosphate, all showed similar sensitivity to acid and displayed equal growth stimulating activity toward *Lactobacillus casei*.

Lichstein & Cohen (141) have shown high transaminase activity (aspartic-glutamic) in a number of bacterial species. Lichstein, Gunsalus & Umbreit (142) have grown *Strep. fecalis* R highly active in transaminase activity and from it have obtained cell-free transaminase preparations. On ageing and dialysis some destruction of coenzyme occurred and activity could be restored by addition of pyridoxal phosphate. Cells grown in pyridoxal-deficient medium gave preparations of the apoenzyme which could be brought to full activity by the addition of pyridoxal phosphate.

Snell (143) has found that when pyridoxal and glutamic acid are heated together they undergo a reversible transamination with the

formation of pyridoxamine and α-ketoglutaric acid; other amino acids seem to react similarly. It seems probable that pyridoxal phosphate acts as a prosthetic group in transaminase, alternately accepting and donating amino groups.

Though the existence of *l*-amino acid oxidase is now established, there is still the possibility that oxidation of certain *l*-amino acids may occur by the mechanism postulated by Braunstein & Bychkov (144). These authors suggested that the amino acid is oxidatively deaminated by transamination with α-ketoglutaric acid and that the glutamic acid formed is reoxidised to α-ketoglutaric acid by glutamic dehydrogenase, a pyridine nucleotide-determined enzyme. Braunstein & Asarkh (145) report that dilute kidney or liver suspensions, containing added cozymase, deaminate glutamic acid. Aspartic acid, alanine, cysteic acid, valine, and leucine are also deaminated if α-ketoglutarate is added. The deaminations scarcely occur without added cozymase and the rates of deamination and transamination are compatible with the proposed mechanism. The rates with the amino acids decrease in the order in which they are mentioned and no other amino acids appear to be oxidised by this mechanism. Neither of the transaminases of Green *et al.* (136) were found to be active with valine or leucine.

What appears to be an interesting interaction of carboxylase and transaminase activity has been described by Kritsman (146). Rat liver slices with pyruvate and ammonia produce an increase in amino acid nitrogen in the presence of bicarbonate-carbon dioxide, but not in phosphate buffer unless oxaloacetate is added. Aspartic and glutamic acids and alanine are produced. Kritsman believes that oxaloacetate is formed from pyruvate and carbon dioxide. The oxaloacetate is aminated by ammonia to give aspartic acid. The latter, by transamination to pyruvate or to α-ketoglutarate (formed by the tricarboxylic cycle), produces alanine and glutamic acid. Added α-ketoglutarate can, to some extent, replace oxaloacetate in the reaction without carbon dioxide.

INTERRELATIONS OF OXIDATIONS AND PHOSPHORYLATIONS

The reversibility of the phosphoroclastic splitting of pyruvate to acetyl phosphate and formate was mentioned in previous sections. Studies by Potter (147) on the coupling between phosphorylation and the oxidation of the C_4 acids were reported in the previous review (94).

The apparent irreversibility in biological systems (148) of the reaction

$$\text{Phosphopyruvate} + \text{adenosinediphosphate} \rightleftharpoons \text{pyruvate} + \text{adenosinetriphosphate}$$
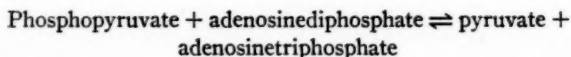
has led to considerable speculation concerning the first step in the synthesis of carbohydrate from pyruvate and lactate via a reversal of the Embden-Meyerhof scheme (103, 149). Lardy & Ziegler (150) have now demonstrated the reversibility of this reaction in muscle extract. Labeled phosphorus, incorporated into ATP during the oxidation of glyceraldehyde-3-phosphate, came into equilibrium with the phosphorus of phosphopyruvate. Pyruvate was enzymatically phosphorylated when a supply of high energy phosphate was maintained by the oxidation of glyceraldehyde-3-phosphate. The reaction was dependent upon the presence of potassium ion.

The activity of the enzyme, choline acetylase, which causes the synthesis of acetylcholine in the presence of ATP (151, 152) has been shown to require potassium ions (72). Nachmansohn & John (83) find activating effects of citrate, cysteine, glutamate, alanine, and cyanide; the C_4 dicarboxylic acids are without effect and α-keto acids are inhibitory. Feldberg & Mann (153) find effects with citrate and boiled brain extract.

Lipmann (154) has prepared pigeon liver suspensions and extracts which cause the acetylation of sulfanilamide and also, less actively, of other sulfonamides and p-aminobenzoic acid. With suspensions, acetylation took place aerobically and the rate was increased if acetate or, less actively, acetoacetate, pyruvate, acetoin, or diacetyl was added. Anaerobically or with cell-free extracts, the acetylation was slight but it could be largely restored by the addition of ATP. No evidence was obtained that acetyl phosphate is active in the condensation, and Lipmann suggests that acetate, sulfanilamide, and ATP form a complex in the presence of the enzyme which breaks up into acetylsulfanilamide, adenylic acid, and inorganic phosphate.

Lehninger (101, 102) found that fatty acids are oxidised by liver preparations in the presence of adenosinetriphosphate and concluded that formation of acyl phosphate precedes oxidation. By adding hydroxylamine, to capture acyl phosphate as hydroxamic acid before it could be decomposed by acyl phosphatase, Lipmann & Tuttle (155) have been able to show that pigeon liver extracts produce acetyl phosphate from acetate and ATP. With relatively high hydroxylamine

concentrations, a general reactivity of fatty acids with pigeon or rat liver suspensions occurs independently of added phosphate donor or the presence of oxygen.

Klein (156) reports that the oxidation of $l(+)$-glutamic to α-ketoglutaric acid by brain extracts fortified with cozymase and other additions, may be coupled with phosphorylation of glucose. Long (157) has shown that dialysed ground pigeon-brain suspension causes the oxidation of added fumarate or malate to oxaloacetate or pyruvate (not distinguished), aerobically or with methylene blue as hydrogen acceptor. The keto acid formation is strongly catalysed by inorganic phosphate but unaffected by adenine nucleotide. Malonate is strongly inhibitory.

The fixation of inorganic phosphate in the aerobic metabolism of kidney slices has been shown by Kaplan et al. (158).

Stone et al. (159) and Gurdjian et al. (160) found that under conditions of anoxia or experimental head injury, the brain content of phosphocreatine and ATP decreased. Proger et al. (161) find a decrease in easily hydrolysable phosphate in heart and kidney following reduced oxygen supply in vivo. Intravenous injections of cytochrome-c largely prevented this, but the mechanism of the effect is not clear. From determination of a number of blood constituents, including the breakdown products of ATP, McShan et al. (162) conclude that catabolic changes prevail and energy reservoirs are depleted in the tissues during the development of shock initiated by various procedures.

McElroy & Ballentine (163) have found that purified luciferin contains acid-labile phosphate which is partly changed to inorganic phosphate after the luminescent reaction. They propose a scheme for the bioluminescence reaction which is like the reverse of the present concept of photosynthesis. The side chain, $R \cdot CO \cdot CH_2OH$, is oxidatively degraded through four steps to $R \cdot COOH$ releasing six hydrogen atoms and one carbon dioxide. The energy represented by the hydrogen atoms is preserved as phosphate bond energy and released during luminescence.

CELL AND TISSUE METABOLISM

In spite of the large amount of work which has been done with tissue slices or other whole tissue preparations, methods have not yet been so standardized that accurate duplication of results can be ob-

tained or that small differences may be reliably detected between normal and abnormal tissues. Studies by Fuhrman & Field (164) have been directed toward such standardization. With liver slices the rate of respiration is a linear function of temperature between 25 and 45°C. The optimum slice thickness is 0.48 to 0.62 mm. in oxygen. With thicker slices the rate is presumably limited by diffusion of oxygen into the slice. With thinner slices, as with slices subdivided into smaller pieces, damage to cell structure, disrupting respiratory systems, is greater. The rate per unit weight varies inversely with the glycogen content presumably because glycogen, with the water associated with it, acts as a diluent of the active liver tissue. Fuhrman & Field (165) found that the respiration rate of liver, kidney, and brain slices was decreased by keeping the whole tissue at 35°C. before slicing. With liver and kidney the effect of such a period of inadequate oxidation was minimised at 10°C. Bernheim & Bernheim (166) found similar but more marked effects with heart.

Elliott & Henry (167) find that isotonic brain suspensions continue to respire at maximum rate at oxygen tensions as low as 4 mm. Hg. Glycolysis sets in at lower tensions, but it occurs at maximum rate in the presence of traces of oxygen, and not in complete anaerobiosis. Slight aerobic metabolism of glucose is sufficient to maintain a pyruvate concentration approaching that required (5×10^{-5} molar) for maximum stimulation of glycolysis. Using cytolysed suspensions and extracts, Utter, Wood & Reiner (168) have made a careful study of the factors involved in the determination of the total potential activity of the glycolytic enzyme systems of brain. The Geiger inhibitor of glycolysis was shown to be a diphosphopyridine nucleotidase. Rosenthal *et al.* (169) found that brain suspensions from animals subjected to temporary arrest of cerebral circulation showed, in some cases, a decreased carbohydrate oxidation, but there was no essential change with brains from animals subjected to carbon monoxide hypoxia or hemorrhagic shock.

Slowly developing inhibition of brain tissue respiration by one atmosphere of oxygen had been observed by Quastel (170) and Elliott & Libet (171). Stadie, Riggs & Haugaard (172) find that the respiration of slices of brain from animals killed by high oxygen pressure, eight atmospheres, seemed normal. When brain slices or suspensions were subjected to high oxygen pressure, a slowly developing inhibition of respiration occurred. The extent depended upon the oxygen pressure and time of exposure and was not reversible. The

inhibition did not involve a change from the normal predominantly carbohydrate character of the metabolism. Similar, but more slowly developing, inhibitions of respiration were observed with kidney, liver, lung, and muscle tissue. The slowly developing effects on tissue metabolism, or on isolated systems mentioned earlier, could not account for the rapid development of nervous symptoms and early death of animals subjected to high oxygen tension.

Warren (173) finds that the rate of respiration of bone marrow, liver, and kidney cortex, in bicarbonate-containing medium is usually higher than in phosphate buffered medium. The higher rate could be shown in phosphate medium containing initially $0.003\,M$ or less bicarbonate, by simple manometry in which evolved carbon dioxide is absorbed by alkali. Under these conditions the bicarbonate slowly disappears but the effect on respiration, once initiated, remains. Serum and its ultrafiltrate contain other factors besides bicarbonate which affect respiration.

Barron & Huggins (174) have studied the metabolism of prostatic tissue. The respiration and pyruvate oxidation are decreased and aerobic glycolysis is increased after castration of the animal or injection of stilbesterol. Lan & Sealock (175), pursuing the studies of Sealock on the role of ascorbic acid in controlling the metabolism of aromatic amino acids, have found that the respiration of normal guinea pig liver slices is increased in the presence of l-tyrosine but this does not occur with tissue from scorbutic animals; the effect is restored if the vitamin is added to the medium. On the other hand, Darby et al. (176) find that liver slices from both scorbutic and normal animals produce a hydroxyphenyl compound from phenylalanine and metabolise tyrosine as indicated by disappearance of hydroxy groups. It appears that lack of ascorbic acid involves inability of the liver to oxidise the side chain of tyrosine.

The metabolism of the parasitic helminth, Ascaris suis, has been studied by Laser (177). The respiration of these organisms increases with oxygen tension but they are killed within an hour by one atmosphere of oxygen. They survive anaerobiosis for many hours but develop an oxygen debt. The respiration is low, apparently due, among other factors, to a low cytochrome content and to an accumulation of oxaloacetic acid which inhibits succinic dehydrogenase. The toxicity of high oxygen tension is probably due to the hydrogen peroxide which accumulates. The catalase activity is very low. The worms are not killed by $0.01\,M$ cyanide and neither cyanide nor azide has

any immediate effect on the respiration of the whole worm or muscle pulp from it.

The oxygen consumption of *Paramecia,* particularly when glucose is supplied, is partially inhibited by cyanide. Starved specimens are insensitive to cyanide (178).

With six types of yeast Spiegelman & Nozawa (179) have confirmed earlier work of Stier & Stannard (180, 181) in finding that consumption of carbohydrate reserves in yeast occurs by purely aerobic processes not involving a preliminary fermentative process. Without external substrate there is no fermentation aerobically or anaerobically.

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NON-OXIDATIVE ENZYMES

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Proteolytic Enzymes

An extensive account of recent work on enzymes which hydrolyze the peptide linkage was given by Greenberg & Winnick in last year's *Annual Review of Biochemistry* (1). The present discussion of these enzymes is necessarily limited to a consideration of a relatively small number of contributions selected from the more recent literature.

Plant proteinases.—To the list of these enzymes previously recorded (1) may now be added the proteinase "arachain" which was isolated in purified form from the cotyledons and embryo of the peanut by Irving & Fontaine (2). This enzyme functions optimally at pH 6.5 to 7.5 and its activity is not influenced by cyanide, cysteine, or hydrogen peroxide when these compounds are present in concentrations which have a marked effect on several other proteinases. Moreover, the hydrolysis of the synthetic substrate benzoyl-*l*-arginine amide was shown to proceed in the first stages as a reaction of zero order, even at very low concentrations of substrate. The authors point out that this behavior differs from that of other proteolytic enzymes whose reaction kinetics have been studied. In practically all other cases the kinetics of the hydrolysis of synthetic substrates are those of first order reactions. Castañeda and co-workers (3) have succeeded in crystallizing the enzyme mexicain which is found in the latex of *Pileus mexicanus*.

Digestion of native and denatured proteins.—The fact that denatured proteins are, in many instances, more readily hydrolyzed by enzymes than the corresponding native proteins seems now to be well established. In a comparative study of the papain-digestion of native human serum albumin and of this protein after denaturation by heat and by urea, Rice and co-workers (4) have observed some interesting effects of sodium caprylate and of the sodium salts of other acids such as phenyl butyric acid on the denaturation process. Each of these salts not only protects native serum albumin from undergoing coagulation at a temperature at which the protein is quickly coagulated in the absence of the salt; but the salts also have the effect of "stabilizing" the protein during treatment by urea or by heat in such a man-

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ner that the rate of subsequent hydrolysis by papain is not appreciably different from the rate of hydrolysis of native protein. In the absence of stabilizing salt, either urea denaturation or heat treatment renders the protein more susceptible to papain-hydrolysis, urea being much more effective than heat in this respect. The effect of urea is largely abolished if the urea is subsequently removed by dialysis; the albumin apparently undergoes regeneration as the result of the removal of urea and is then hydrolyzed by papain at a rate which is only slightly higher than that at which native albumin is hydrolyzed. The mechanism of the action of sodium caprylate in protecting serum albumin against denaturation by heat or urea is not known. The authors suggest that the liberation of active groups or the extension of the molecule by urea or by heat may be prevented by the salt. It is not improbable that the liberation of free sulfhydryl groups as an accompaniment of denaturation may be concerned with the greater rate of papain-hydrolysis of the protein after denaturation. In this connection the recent work of Harington & Rivers (5) is of considerable interest. Studies of the relative rates of hydrolysis of tyrosylcysteine and tyrosylcystine, of cysteyltyrosine and cystyltyrosine, and of their N-carbobenzyloxy-derivatives, by crystalline pepsin, indicated that the presence of a sulfhydryl group in the neighborhood of the peptide bond greatly accelerates the rate of cleavage. Substitution of the free sulfhydryl group of N-carbobenzyloxycysteyltyrosine by the -S-benzyl group abolishes the activating effect. Harington & Rivers suggest that the increased rate of the peptic hydrolysis of proteins after denaturation may be related to the liberation of sulfhydryl groups during denaturation: these groups may activate the cleavage of peptide linkages in the protein similar to those in the synthetic peptides which they investigated.

Haurowitz et al. (6) have noted a difference between fibrous and globular proteins with respect to the effects of denaturation on susceptibility to hydrolysis by purified trypsin. Fibrous proteins such as fibrinogen and myosin were hydrolyzed at the same rate before and after denaturation, whereas the globular proteins, egg albumin and serum globulin, were hydrolyzed more quickly after denaturation. It was suggested that denaturation of globular proteins results in an unfolding of closely packed peptide chains and has the effect of making accessible to the enzyme the atomic groupings in the protein molecule which serve as points of attack by the enzyme. According to Harte (7) fresh egg white and commercial samples of dried egg white

are both resistant to the action of trypsin and contain similar amounts of an anti-tryptic principle; coagulated egg white is rapidly hydrolyzed by trypsin. Peptic hydrolysis of fresh and dried egg white is somewhat slower than that of the coagulated material. The rate of digestion of the acid-insoluble proteins of milk by gastric, duodenal, and pancreatic enzymes was found by Turner (8) not to be appreciably affected by pasteurization, homogenization, or desiccation of the milk, but it was increased when whole milk was heated to relatively high temperatures.

Trypsin and chymotrypsin.—In studies of the effects of heparin, hexylresorcinol, and pancreatic trypsin-inhibitor on the activities of trypsin and of chymotrypsin in the early stages of casein hydrolysis in which the formation of a precipitate unaccompanied by any change in formol titration value was observed, and in the later stages in which increased formol titration values become apparent, Horwitt (9, 10, 11) noted differences between the two proteinases. For example, heparin inhibited the action of trypsin to a much greater extent than that of chymotrypsin. He pointed out that commercial preparations of "trypsin" probably contain both trypsin and chymotrypsin and therefore the use of such preparations in studies of the antagonism between heparin and trypsin *in vivo* and *in vitro* may lead to considerable confusion. Horwitt believes that the anti-tryptic action of heparin, demonstrable *in vitro,* is probably an unimportant factor in its physiological action since the serum contains another antiproteolytic substance which is much more powerful than heparin.

The activity of chymotrypsin appears to be dependent on the presence of tyrosine in the enzyme molecule. Sizer (12) investigated the effects of oxidizing and reducing compounds and of phenylisocyanate, ketene, nitrous acid, and formaldehyde on the rate of digestion of purified collagen by crystalline chymotrypsin. The fact that the activity of the enzyme was unaffected by changes in the oxidation-reduction potential within the range extending from -400 to $+500$ millivolts was interpreted as indicating that neither the sulfhydryl nor the disulfide group is essential for activity. Inactivation by ketene and by nitrous acid appears to be due to reactions of these reagents with tyrosine groups rather than with free amino groups. Phenylisocyanate which reacts with the latter groups had relatively little effect on the activity of the enzyme.

The nature of peptones.—In studies of the hydrolysis of egg albumin, fibrin, and casein by commercial preparations of pepsin and

by purified pancreatic proteinase free from polypeptidase and pro-
taminase, Fodor and co-workers (13, 14) determined the "quotient
of cleavage," that is the ratio, total nitrogen cleavable / nitrogen split
hydrolytically, after making allowance for that part of the nitrogen
of the hexone bases and of tryptophane which does not enter into the
formation of peptide linkages. This quotient was used as an index
of the extent of hydrolysis. Pepsin and pancreatic proteinase, acting
independently, brought about the same amount of cleavage of egg
albumin and of fibrin, the quotient of cleavage having a value of ap-
proximately four in all four cases; casein was hydrolyzed somewhat
more completely by trypsin than by pepsin, judged on this basis. After
exhaustive digestion with pepsin, additional cleavage by trypsin re-
duced the quotient to a value of approximately three; but partial hy-
drolysis by pepsin followed by final cleavage by trypsin resulted in
a quotient of about four in all cases. A quotient of four was inter-
preted as indicating that peptones are mixtures of open tetrapeptides
formed by cleavage of closed tetrapeptide complexes in the protein
molecule, and that one particular proteinase is unable to hydrolyze
more than one linkage in each closed tetrapeptide. Further degrada-
tion of open tetrapeptides is only possible, according to the authors,
if the tetrapeptide contains linkages of a specific configuration, easily
hydrolyzed by the enzyme.

It is questionable whether the method adopted in this study of
the nature of the products of the peptic and tryptic hydrolysis of pro-
teins is sufficient, by itself, to yield the desired information. It will
be recalled that Tiselius & Eriksson-Quensel (15) reached somewhat
different conclusions regarding the nature of the products of the hy-
drolysis of crystalline egg albumin by crystalline pepsin. In the case
of both partial and prolonged hydrolysis a residue of high molecular
weight remained, and after removal of low-molecular split products
by dialysis, this high-molecular fraction resembled unchanged egg
albumin in its electrophoretic mobility and sedimentation constant.
The physical nature of the low-molecular dialyzable fraction appeared
to be unaffected by the duration of the hydrolysis: the diffusion con-
stant of this fraction was the same after sixty-three hours' as after
three hours' digestion, and estimations of the average molecular
weight from sedimentation equilibria indicated a value of about 1000.
This corresponds to eight amino acids, on the average, in the pep-
tides constituting the dialyzable fraction; since the material was not
homogeneous one cannot assume that it contained only octapeptides.

But the authors concluded that at no stage of hydrolysis was there any appreciable accumulation of products having a molecular weight intermediary between that of unchanged protein and the average molecular weight of the fully digested end-products.

Proteolytic enzymes in burned areas.—In experiments designed to shed some light on the nature of the toxin which is believed to be formed as the result of skin burns, Beloff & Peters (16) discovered in the skin of man, rats, rabbits, and guinea pigs a proteinase having an optimum pH of approximately 7.5, which, they concluded, is not trypsin or chymotrypsin because it does not hydrolyze benzoylarginineamide or carbobenzoxy-*l*-tyrosylglycineamide. After burning at temperatures which were not high enough to cause appreciable heat-inactivation of the enzyme, there was nevertheless a decrease in the proteinase content of the burned area of the skin. It was suspected at first that the liberated enzyme may cause some systemic disturbance but later work indicated that the plasma contains an anti-protease which neutralizes the enzyme (17). Zamecnik *et al.* (18) studied the effect of burning (e.g., immersion of the legs of anesthetized dogs in water at 90°C. for fifteen seconds) on the peptidase activity of the lymph. Using *l*-leucylglycylglycine as substrate they observed an abrupt rise in the peptidase activity of the lymph draining the affected area. A similar enzyme was identified in the blister fluid collected from human burns. In the cat, rat, and calf, burning of superficial areas caused an increase in the peptidase activity of the serum. A similar enzyme was found in normal dog serum and it increased in amount after burning. There is no evidence to indicate that the increased peptidase activity is directly related to the toxic effects of burns.

Aminopeptidase.—Ågren (19, 20) has reported further observations on the nature and properties of the aminopolypeptidase which he extracted from dried pyloric mucosa of hogs and which he believes to be identical with the intrinsic anti-anemic factor of Castle. He obtained a preparation which was practically free from dipeptidase and carbohydrate, and which did not digest highly purified liver preparations containing the anti-pernicious anemia principle. The presence of a heavy metal in the preparation was indicated by the inactivating effects of cyanide, cysteine, and pyrophosphate.

Streptococcal fibrinolysin.—Cultures or culture-filtrates of certain strains of β-hemolytic streptococci are known to cause rapid lysis of the fibrin clot formed on adding calcium or thrombin to human

plasma. This phenomenon is known as fibrinolysis and has been attributed to an agent called fibrinolysin produced by the bacteria. Fibrinolysis seems to be associated, among the hemolytic streptococci, with the Lancefield groups A, C, and G in particular, and is not shown by organisms of groups B, D, F, and H even though they are derived from human sources (21, 22). The exact nature of the fibrinolytic reaction has been a matter of considerable speculation and it is only recently that it has been shown to be proteolytic in character.

Mirsky (23) observed that the anti-fibrinolysin which develops in human plasma in β-hemolytic streptococcal diseases runs parallel with the anti-tryptic factor. He demonstrated that crystalline trypsin-inhibitor isolated from beef pancreas or a trypsin-inhibitor prepared from soybean, when added to a saline mixture containing human fibrinogen, thrombin, and streptococcal fibrinolysin, delayed the liquefaction of the clot to such an extent as to indicate complete inhibition of fibrinolytic activity. On these grounds he suggested that fibrinolysin is a protease and may be related to trypsin.

Christensen (24, 25), however, regards streptococcal fibrinolysin as an activator of an inactive proteolytic enzyme, or zymogen, which is present in human serum, and he believes that the relationship of the two factors to each other is similar to that of trypsinogen to enterokinase and of chymotrypsinogen to trypsin. Milstone (26) had concluded that human serum contains a "lysin factor," in the globulin fraction, which is necessary together with fibrinolysin in order to cause fibrinolysis. Christensen (24) confirmed this view and showed that the combination of the lysin factor of the serum with streptococcal fibrinolysin causes not only the lysis of fibrin clots but also the proteolytic degradation of fibrin, fibrinogen, casein, and gelatin. For this purpose he employed measurements of changes in viscosity, of the increase in acid-soluble "tyrosine," and of the increase in amino nitrogen and in total soluble nitrogen. When a constant amount of lysin factor was incubated with varying amounts of fibrinolysin, the total proteolytic activity which ultimately developed was proportional to the time of incubation rather than to the concentration of fibrinolysin. But the rate of activation was proportional to the concentration of fibrinolysin. From these and similar results it was concluded that the activation of lysin factor by fibrinolysin is a catalytic reaction, rather than a stoichiometric reaction between the two substances.

The fibrinolysin-activated lysin factor was shown to resemble in several respects the protease in serum which can be rendered active

by treatment of the serum with chloroform (25). For example, the pH-optima, the pH of maximum stability, and the energy of activation were comparable in the two cases. On the other hand, the activated lysin factor differed from trypsin with respect to optimum pH and to such properties as those which are concerned with the extent of casein hydrolysis and the nature of the linkages split, and with susceptibility to the influence of the trypsin-inhibitors of pancreas and serum. Christensen concluded that "fibrinolysin-activated lysin factor and the chloroform-activated protease of serum are one and the same enzyme, differing only in their mode of activation."

Holmberg (27) had previously reached similar conclusions regarding the mode of action of fibrinolysin. He also believes that the fibrinolytic enzyme exists in human plasma in an inactive state and that fibrinolysin functions as an activator.

If fibrinolysin is merely an activator, the name of the agent is misleading. Christensen (25) suggests that the inactive precursor in the blood might appropriately be called "plasminogen" and the activated enzyme "plasmin," in conformity with the usage for some proteases such as bromelin, ficin, and papain, where the prefix indicates the source of the enzyme.

Miscellaneous studies of proteolytic enzymes.—Reznitschenko *et al.* (28) claimed that they were able to isolate from pancreatin a crystalline enzyme which, when acting on gelatin, causes only the transformation of the gelatin into a form which is soluble in trichloroacetic acid solution, without hydrolyzing any peptide linkages. Roche & Mourgue (29) used the method of Fromageot & Mourgue (30) in a study of the rate of liberation of valine and leucine during the hydrolysis of casein, edestin, globin, and zein, and concluded that the distribution of these amino acids in the proteins does not present a uniform periodicity. Roche & Vialatte (31) stated that papain, pepsin, and trypsin, each acting at its optimum pH, liberate from edestin, casein, and gelatin not only free amino and carboxyl groups but also monosubstituted guanidine groups, and it was suggested that some of the guanidine groups exist in the intact protein in combination with carboxyl groups of dicarboxylic acids. Elliott (32) showed that a number of strains of streptococci of group A produce a proteolytic enzyme which can attack the type-specific protein antigen (the "M-substance"). The general properties of the enzyme resemble those of papain and some of the cathepsins. Certain forms of lignin and tannin have been used by Wallerstein *et al.* (33) to precipitate

the protease from extracts of molds. The enzyme preparation was stable for several months in the dried form and was freely soluble. Berridge (34) described more fully the preparation and properties of crystalline rennin, the isolation of which was reported earlier. Rennin was found by Dotti & Kleiner (35) to be absent from adult gastric juice. For the measurement of the rate of hydrolysis of peptide linkages, Zamecnik et al. (36) developed a photometric adaptation of Linderstrom-Lang's method of titration in an acetone medium. The sensitivity of the modified method is such that 2.8×10^{-8} mole of leucylglycine can be titrated with considerable accuracy. A protein having trypsin-inhibitory properties was prepared in crystalline form from an acid extract of soybean meal by Kunitz (37). It is a protein of the globulin type, free from carbohydrate, and has very little influence on the proteolytic and milk-clotting activities of chymotrypsin. According to Cohen (38) crystalline ribonuclease has a marked proteolytic effect on thymus nucleohistone, as indicated by the liberation of tyrosine and arginine. Zamecnik & Stephenson (39) observed that the catheptic activity of the cortex of hog kidney is higher than that of the medullary region.

Non-Proteolytic Enzymes

Arginase.—In view of the much lower rate at which arginase hydrolyzes a definite amount of arginine when the latter is present in a protein hydrolysate, in comparison with the rate of hydrolysis of an equivalent amount of arginine in a mixture containing no other amino acids, Hunter & Downs (40) have investigated the inhibitory effects of a large number of amino acids and amino acid derivatives and of some proteins, on the activity of purified liver arginase at pH 8.4. In concentrations up to 2.5 per cent casein and gelatin were without effect and egg albumin was only slightly inhibitory. When present in concentrations up to 0.089 M, urea had no effect on the enzyme. The activity of the enzyme was, however, inhibited in varying degree by all the α-amino acids investigated, having the *l*-configuration, but not by the *d*-isomers or by monoamino acids having the amino group in the β-, δ-, or ξ-position. Of the α-amino acids, only ornithine and lysine exhibit a clearly competitive type of inhibition; the monoamino acids exercise their inhibitory effects in an essentially non-competitive manner, though in some cases there was evidence of a minor degree of competitive inhibition also. For both competitive and non-competitive inhibitors the relation between the

concentration (I) of the inhibitor and α, the activity of the enzyme expressed as a fraction of its activity in the absence of inhibitor, is given as $I.\alpha/1\text{-}\alpha = C$, where, at any one concentration of substrate, C is a constant which is characteristic for each amino acid. In the case of the competitive inhibitors, ornithine and lysine, the following relationship exists: $C = K_i + (K_i/K_a).A$, where K_i and K_a are the dissociation constants of inhibitor-arginase and arginine-arginase, and A is the molar concentration of arginine. Calculations of the constants yielded values of 0.01 M for K_a, not more than 0.004 M for K_i (ornithine), and not less than 0.0048 M for K_i (lysine); the enzyme has, therefore, a considerably higher affinity for each of the competitive inhibitors than for the substrate. The effects of the non-competitive inhibitors vary in magnitude and appear to depend partly upon the length and branching of the carbon chain and partly upon the nature and position of substituent radicals.

Kochakian has continued his studies of the effects of steroid hormones, implanted subcutaneously in mice after castration, on the arginase content of the kidney. He had previously observed (41) that although the weight of the kidney is decreased as the result of castration, its total arginase content is not appreciably affected; the implantation of certain steroids after castration is followed by a marked increase in total arginase, particularly in the case of testosterone and 17-methyltestosterone, whereas other steroids reduce the amount of the enzyme and still others have no significant effect. Further work (42) has indicated that the difference in arginase activity obtained among steroids causing maximum response in the size of the kidney disappears when the comparison is made on the basis of the amount of steroid absorbed. Testosterone, testosterone propionate, androstanediol-3(α),17(α), and androstanol-17(α)-one-3 produce almost identical responses per mole of steroid absorbed. The 17-methyl derivatives of testosterone and androstanediol-3(α),17(α) cause a more rapid initial increase in kidney arginase than do the latter compounds themselves. The author points out that only the enzymes (acid and alkaline phosphatase, d-amino acid oxidase, and arginase) of the kidney, and not those of the liver or intestine, are known to be affected by steroid hormones. It is difficult, as yet, to assess the significance of this work on kidney arginase in relation to the influence of steroid hormones on the intermediary metabolism of arginine and of proteins.

A study of the effects of metallic ions on the activity and stability

of purified liver arginase was reported in abstract form by Mohamed & Greenberg (43). Activation by calcium and manganese ions was found to be a reversible reaction which is influenced by time, temperature, pH, and by the type and concentration of the activating ion. In solution, arginase was found to be quite stable even in the presence of most metallic ions except silver and mercury which inactivate it. The enzyme was destroyed by iodine but not by hydrogen peroxide.

Anderson (44) found that the activity of arginase in a jack-bean preparation which also contained urease was augmented by cobalt, manganese, or ferrous ions. In the case of cobalt and iron excessive amounts of added ion greatly depress the activity, but only slight depression is caused by excess manganese. The optimum pH increases with rising concentration of cobalt but is independent of changes in manganese concentration. Ferrous ions cause activation only in relatively high concentration at pH 8.8 and are inhibitory at pH 8.2; cysteine augments the activation caused by iron.

Synthesis of urea.—According to Leuthardt & Glasson (45) the ability of the liver of thiamine-deficient rats to synthesize urea in mixtures containing phosphate, bicarbonate, ornithine, and ammonium ions is significantly increased by added thiamine. Riboflavin, nicotinamide, and ascorbic acid are without effect; and the mono- and pyrophosphates of thiamine are less marked in effect than thiamine, probably because of slower diffusion into the regions of enzymatic activity. The authors believe that this effect of thiamine is closely related to the metabolism of pyruvic acid but the specific reaction affected, among the several reactions involving pyruvic acid which are affected by cocarboxylase, is not known.

Bernheim & Bernheim (46) observed that though caffeine does not influence the activity of liver arginase *in vitro* or the deamination of amino acids by liver slices it nevertheless inhibits the formation of urea from ammonia in liver slices. The disappearance of ammonia in slices of both liver and kidney is inhibited by caffeine; the authors attribute the result, in the case of the liver, to decreased formation of urea and of amino nitrogen and, in the kidney, to decreased formation of amino nitrogen.

It has been known for some time that the lower analogue of arginine, namely, α-amino-γ-guanidino butyric acid, can be hydrolyzed by liver preparations to yield α,γ-diaminobutyric acid and urea. Boulanger & Bertrand (47) have synthesized this diamino acid (the lower analogue of ornithine) and have tested its ability to replace

ornithine in urea synthesis, using the technique of Krebs. Since no effect on urea formation was observed, it was concluded that the presence of ornithine is a specific requirement for synthesis.

The previously reported formation of urea from glutamine under the influence of a cell-free extract of liver (48) was shown by Archibald (49) to have had its origin in arginine present as an impurity in the glutamine.

Histidase.—Although the product of the action of this enzyme on histidine has not yet been isolated, Walker & Schmidt (50) suggest that it is α-formamidino glutaric acid,

$$COOH \cdot CH_2 \cdot CH_2 \cdot CH(NH \cdot CH : NH) \cdot COOH.$$
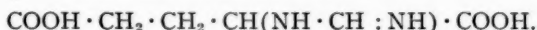
They believe that the formation of this compound would be consistent with the following observations which they made in a study of the action of purified histidase, prepared from cat liver, on histidine: no change in acidity during the reaction, formation of a new group with a pK' value of 4.2 (possibly a second carboxyl group), retention of optical activity, loss of α-amino nitrogen, lability towards alkali, and considerable stability in acid solution. The activity of histidase when determined at various pH levels in the presence of excess substrate showed a sharp optimum at pH 8.5. The acid branch of the pH-activity curve fitted closely the following equation (equation 1): $P = V_m t/(1 + [H^+]/K_{ES})$ where P represents the product formed, K_{ES} is the dissociation (ionization) constant of the enzyme-substrate complex, and V_m is a measure of the concentration of enzyme present, expressed in moles of substrate degraded per liter per hour when t is expressed in hours. The course of the histidase reaction was shown to follow the equation (equation 2), $V_m = P + K_m \cdot ln[A/(A\text{-}P)]$ where A represents total substrate added and K_m the Michaelis constant. When A is much larger than $V_m t$ and K_m, the relationship in equation 2 becomes $V_m t = P$ and, therefore, under these circumstances, measurements of P and t will permit the calculation of V_m expressed in the terms stated above. The value of K_m was determined from the slope ($-1/K_m$) of the straight line obtained by plotting $1/t \cdot ln[A/(A = P)]$ against P/t (abscissa), the intercept on the P/t axis being V_m; K_m was found to be 1.21×10^{-3} M histidine. The calculated rates of breakdown of histidine at different substrate concentrations did not deviate systematically from the theoretical equation (equation 2). Excess substrate beyond that necessary to saturate the enzyme did not appear to be inhibitory.

Thrombin.—Although this enzyme acts specifically on the protein fibrinogen there is, as yet, no satisfactory proof that its action is proteolytic in the sense ordinarily associated with this term; for this reason thrombin is included here among the non-proteolytic enzymes. The detectable chemical differences between fibrinogen and fibrin are, indeed, so slight that the mechanism of the enzymatic transformation of the former into the latter has been, to a great extent, a subject of speculation only. Baumberger's suggestion (51) that the change is one of oxidation of sulfhydryl groups in fibrinogen to form —S—S— linkages in the resultant fibrin gains support from some interesting observations of Lyons (52, 53) which were published subsequently to the preparation of Chargaff's review (54) of the recent literature relating to the coagulation of blood. Space does not permit a detailed discussion of Lyons' experiments; only his main conclusions can be stated. He believes that thrombin contains two components, A and B, having separate functions, and that both components are necessary for the conversion of fibrinogen into fibrin. Thrombin A catalyzes the liberation of —SH groups which are normally blocked in the fibrinogen molecule. Changes in the curves obtained in the polarographic analysis of purified fibrinogen after a short period of treatment with thrombin were interpreted as indicating the formation of fibrinogen-SH as an intermediary between fibrinogen and fibrin. Lyons believes that the B-component of thrombin then converts fibrinogen-SH into fibrin. Fibrin is regarded not merely as a compound formed by the union of two fibrinogen molecules through an —S—S— linkage, but rather as a long chain of such molecules giving rise to the typical gel structure. Transformation of fibrinogen-SH into a fibrin gel could also be brought about by the addition of minute amounts of 2-methyl-1,4-naphthoquinone. The products of the partial hydrolysis of thrombin by trypsin were shown to contain a substance which reacts with 2,4-dinitrophenylhydrazine to give the characteristic green color which is said (55) to be given only by naphthoquinone and its derivatives. Lyons suggests that either vitamin K is a functional prosthetic group of thrombin B or it converts a protein fraction into an oxidation-reduction system having a potential near that of vitamin K.

From the results of studies of the influence of pH on the clotting process, Laki & Mommaerts (56) also conclude that the conversion of fibrinogen into fibrin takes place in two steps. They believe that the conversion of the primary product into fibrin is possible only when

fibrinogen is negatively charged. The primary product, on the other hand, can be formed when thrombin and fibrinogen react at pH levels on the acid side of the isoelectric point of fibrinogen as well as on the alkaline side.

A paper of considerable interest is concerned with the effects of penicillin on the coagulation of the blood (57). The oral or intramuscular administration of penicillin to human subjects was followed in a few minutes by a marked reduction in the clotting-time of the blood. As the concentration of penicillin in the blood rose, the clotting-time fell and remained at low levels even after penicillin had disappeared from the blood. Moreover, the clot which formed was non-retractile. The authors emphasize the possible danger of thrombus formation following the administration of large amounts of penicillin and they suggest that the drug may have some value as a coagulant in hemorrhagic disorders.

Hydrogen sulfide-liberating enzymes.—Since the publication of Smythe's review (58) which includes a discussion of these enzymes, some additional papers from Fromageot's laboratory have become available. The French workers use the names "cysteinase" and "desulfurase" to designate enzymes in bacteria and in animal tissues, respectively, which catalyze the liberation of hydrogen sulfide from cysteine, but, as they believe, in somewhat different reactions.

Studies of the inhibitory action of a variety of compounds on the cysteinase activity of *Bacillus subtilis* (59) and on the desulfurase activity of animal tissues (60) led Fromageot and co-workers to conclude that both enzymes contain three active groups: a carbonyl group, a carboxyl group, and a basic nitrogen group. The first of these, it was suggested, constitutes the point of attachment of the sulfhydryl group of cysteine while the other two are regarded as activating groups. Homocysteine is believed (61) to inhibit desulfurase activity by competition with the substrate for the carbonyl group of the enzyme whereas glycollic acid and serine inhibit non-competitively by blocking the activating groups. Chaix & Fromageot (62) have found that the desulfurase activity of liver preparations is greatly reduced or even abolished in the presence of oxygen. They emphasize the importance of this effect of oxygen because they believe that it represents a significant difference between the cysteine desulfurase of higher animals and most of the bacterial cysteinases; the activity of the latter is often greater, it is said, under aerobic than under anaerobic conditions.

At the present time it is difficult to reach a decision on the question as to a difference in the nature of the reactions catalyzed by the bacterial and tissue enzymes. Fromageot believes that the two reactions are not identical, whereas Smythe is inclined to believe that they are essentially similar. It would seem that only by the use of much more highly purified preparations of the enzymes will it be possible to settle the question. The use of purified enzymes, free from other enzymes which catalyze side reactions, will also permit more valid conclusions as to the nature of the active groups of the enzymes.

Greenstein & Leuthardt (63 to 66) found in mammalian liver, kidney, and pancreas, an enzyme "exocystine desulfurase" which catalyzes the removal of hydrogen sulfide from cystine-peptides in which cystine occupies either of the terminal positions. The formation of ammonia and pyruvic acid in addition to that of hydrogen sulfide was interpreted as indicating a sequence of three reactions: (a) the exocystine peptide, under the influence of exocystine desulfurase, gives rise to the corresponding α,β-unsaturated dehydropeptide, namely, the peptide of dehydroalanine (α-amino acrylic acid), together with hydrogen sulfide and sulfur; (b) the dehydropeptide is hydrolyzed by dehydropeptidase which accompanies the other enzyme in the tissue extracts, and α-amino acrylic acid is liberated; (c) the latter compound is then hydrolyzed spontaneously, yielding pyruvic acid and ammonia. The desulfuration appears to be the slower of the two enzymatic reactions and is, therefore, the rate-limiting process in the degradation of the cystine-peptides. In contrast with the normal tissues which contain both enzymes, all neoplastic tissues so far examined are completely lacking in both exocystine desulfurase and dehydropeptidase.

Polysaccharide phosphorylases.—The purification, properties, and mode of action of plant and animal phosphorylases which catalyze the reversible transformation of glucose-1-phosphate into polysaccharides have been investigated in several laboratories.

Further studies of the properties and inter-relationships of phosphorylases-*a* and *b* of muscle were reported from Cori's laboratory (67 to 70). Although a prosthetic group essential for the activity of phosphorylase-*a* can be removed from the enzyme by treatment with trypsin or with an enzyme present in muscle, the chemical nature of the prosthetic group has not yet been established with certainty. The possibility that it is adenylic acid is suggested by the fact that the very weak activity of crystalline phosphorylase-*b* can, by the addition of

adenylic acid, be made to approach that of crystalline phosphorylase-*a* (70). On the other hand, efforts to identify adenylic acid or pentose as a product of the enzymatic transformation of phosphorylase-*a* to *b* were unsuccessful (68). This conversion from an active to an inactive form takes place, *in vivo,* during muscular activity and was shown (69) to be a reversible process, complete restoration of phosphorylase-*a* taking place after a short period of rest. It was suggested that the temporary inactivation of phosphorylase-*a* during muscular activity "may represent a regulatory mechanism which would prevent the exhaustion of glycogen stores during fatigue."

Detailed procedures for the purification of potato phosphorylase were described by Chaudhury (71) and by Meyer & de Traz (72); Bernfeld *et al.* (73) outlined an enzymatic method of preparing glucose-l-phosphate.

Hidy & Day (74) concluded that the activation of the reaction in which amylose is synthesized by potato phosphorylase is not dependent on the priming action of branched chain polysaccharides though these compounds do serve as activators. Partially hydrolyzed polysaccharides of the amylose type, both synthetic and natural, were found to possess activating properties. Acid hydrolysis of cornstarch increased the amount of activating substance up to a maximum corresponding to the state of aggregation which no longer gives a color with iodine; further hydrolysis of the dextrins beyond this point reduced their activating power. The authors concluded that the activator for potato phosphorylase need not contain more than seven or eight glucose units per molecule. In confirmation of the views of Sumner *et al.* (75), Kuzin & Ivanov (76) believe that the nature of the polysaccharide synthesized by potato phosphorylase depends on the nature of the activating polysaccharide; they have stated that if a small quantity of glycogen is used as the primer the resultant polysaccharide resembles glycogen rather than starch. Cori *et al.* (77) showed, on the other hand, that even though glycogen was used as the activator for muscle phosphorylase the products synthesized in different experiments had an average chain length of about two hundred units. The enzymatic mechanism responsible for the synthesis, *in vivo,* of the branched structure, muscle glycogen, has not yet been explained. But the accumulating evidence supports the view of Cori *et al.* (77) that the formation of branched chain structures is the result of the simultaneous action of two different enzymes, one being a phosphorylase which is specific for α-1,4-glucosidic linkages and the

other an enzyme which catalyzes the formation of 1,6-glucosidic linkages. Brain, heart, liver, and yeast appear to contain both enzymes; it is probable that potato tubers do also, since Haworth *et al.* (78) isolated a product closely resembling amylopectin, which was formed as the result of the action of a potato extract on glucose-1-phosphate. A detailed description of this work and of later studies of the enzymatic synthesis of amylopectin was given by Bourne and co-workers (206, 207).

Disaccharide phosphorylase.—Partially purified phosphorylase prepared from dried cells of *Pseudomonas saccharophila* was shown by Hassid *et al.* (79) to catalyze the synthesis of sucrose in mixtures containing glucose-1-phosphate and *d*-fructose. The synthesis of another non-reducing disaccharide was similarly achieved (80) by allowing a solution of glucose-1-phosphate and *l*-sorbose to incubate in the presence of the bacterial phosphorylase. The sugar was isolated in crystalline form and appeared to be α-*d*-glucopyranoside-α-*l*-sorbofuranoside. Doudoroff (81) has discussed the functions of phosphorylase in the synthesis and utilization of disaccharides by microorganisms and in the synthesis of sucrose by plants.

Nucleoside phosphorylase.—The discovery of a hitherto unknown enzyme in rat liver sheds new light on the mechanism of the degradation and synthesis of nucleosides. Kalckar (82) has made the interesting observation that the enzymatic liberation of hypoxanthine from inosine takes place only in the presence of inorganic phosphate, according to the following reversible reaction: inosine (hypoxanthine riboside) plus inorganic phosphate ⇌ hypoxanthine plus ribose-1-phosphate. The reaction proceeds completely to the right if the liberated purine is oxidized to uric acid by xanthine oxidase; one mole of uric acid is then formed for each mole of inorganic phosphate which disappears. Kalckar (83) has considered the metabolic significance of the reversible reaction and the position of ribose-1-phosphate as a key substance in the biological synthesis of ribose nucleosides.

Transphosphorylations.—On the basis of tracer studies with P^{32} Meyerhof *et al.* (84) concluded some years ago that cozymase takes no part in phosphate transfer in the coupled reactions leading to the formation of 3-phosphoglyceric acid from 3-phosphoglyceraldehyde, and that it serves only as a hydrogen transporter. Recent observations of Colowick & Price (85) indicate that dihydrocozymase may have another function in carbohydrate metabolism in addition to its function in oxidation-reduction reactions. They observed that the

transfer of labile phosphate from adenosinetriphosphate (ATP) to glucose and to fructose-6-phosphate in muscle extracts occurred only in the presence of dihydrocozymase; oxidized coenzyme was without effect. The authors suggested that this activity of reduced cozymase may be related to the Pasteur effect since under aerobic conditions the lowered ratio of reduced to oxidized cozymase would be associated with a depression of the rate of formation of fructose-1,6-diphosphate in the early stages of glycolysis. Price & Colowick (86) noted, further, that inorganic phosphate, in addition to dihydrocozymase, is necessary for the phosphorylation of glucose and of fructose-6-phosphate by ATP in extracts of rat muscle. They suggested (87) that inorganic phosphate is necessary for the formation of guanine, from a precursor of large molecular size, in a reaction similar to that described by Kalckar (82). Dialyzed muscle extracts containing ATP and dihydrocozymase were unable to phosphorylate glucose but were fully activated by added guanine, though not by adenine. The mechanism of this coenzyme-like action of guanine is not known. Further observations on the action of hexokinase of animal tissues in the catalysis of the phosphorylation of glucose by ATP were reported briefly by Price *et al.* (88). They have found that an extract of anterior pituitary, if injected into rats prior to the preparation of the tissue extracts or added to the enzyme preparation *in vitro,* inhibits the hexokinase activity of the tissues, and that this inhibition can be counteracted by insulin either *in vivo* or *in vitro.*

It will be recalled that the results of an experiment in which radioactive phosphate was used led Meyerhof *et al.* (84) to conclude that the direct enzymatic phosphorylation of pyruvate by ATP does not occur in muscle extracts, and that the reaction of phosphopyruvate with adenylic acid to form pyruvate and ATP must, therefore, be irreversible. In this work a dialyzed enzyme preparation was used and was, presumably, free from potassium ions which Boyer *et al.* (89, 90) showed, later, to be necessary for the transfer of phosphate from phosphopyruvate to adenosinediphosphate (ADP). Suspecting that potassium ions are necessary also for the reverse reaction, Lardy & Ziegler (91) investigated this factor and found that the reverse reaction was catalyzed by muscle extracts, but only if potassium ions were present. They showed that P^{32} incorporated into ATP during the oxidation of 3-phosphoglyceraldehyde was in equilibrium with that in phosphopyruvate, and that pyruvate was enzymatically phosphorylated when high energy phosphate (contain-

ing P³²) was continuously supplied by the oxidation of 3-phospho-glyceraldehyde. Since all the other intermediary glycolytic reactions had been shown, previously, to be reversible, it is now possible to regard the transformation of glycogen into lactate, by way of the well established intermediary reactions, as a completely reversible process.

An enzyme catalyzing the dephosphorylation of phosphopyruvic acid by ADP was isolated in crystalline form from human muscle by Kubowitz & Ott (92). The activity of the enzyme was tested in a system containing phosphopyruvic acid, ADP, magnesium ions, dihydrocozymase, and purified lactic dehydrogenase. The presence or addition of potassium was not specifically mentioned but it is not improbable that potassium found its way into the final mixture since a potassium phosphate buffer was used during the purification of the dehydrogenase. Pyruvic acid which was formed in the transphos-phorylation reaction was, in this system, reduced to lactic acid and the resultant change in the light absorption at 334 mµ served as an index of the rate of oxidation of the dihydrocozymase. Under the conditions of the experiment the transphosphorylation reaction was slower than the reduction of the resultant pyruvic acid and therefore the rate of change in the extinction coefficient at 334 mµ could be used as a measure of the speed of transphosphorylation. Activity of the recrystallized transphosphorylase was demonstrated after 190 hours' dialysis against distilled water changed daily. The recrys-tallized enzyme lost only a relatively small part of its activity during lyophilization.

Problems of biological phosphorylation in relation to the trans-formation of energy have been discussed by Meyerhof (93), by Kalc-kar (94), and by Potter (95). Leibowitz & Hestrin (96) have dis-cussed the role of phosphorylases in the alcoholic fermentation of compound sugars, particularly in relation to concepts of direct, as opposed to indirect, fermentation.

Carbonic anhydrase.—The function of carbonic anhydrase in the blood, the purification, crystallization, and properties of the enzyme, and factors affecting its activity *in vitro* and *in vivo* were discussed by Roughton (97) in an account of recent work on the chemistry of carbon dioxide transport by the blood. Davenport (98) found that thiophene-2-sulfonamide is six times more effective than sulfanilamide in inhibiting the carbonic anhydrase of red cells at 0°C. and prob-ably forty times more effective at 38°. He calculated the dissociation constant of the enzyme-inhibitor complex at 0° to be 1.6×10^{-7} in

the case of the former compound and 1.0×10^{-6} for sulfanilamide. The value of K_m in carbon dioxide-uptake experiments, in the absence of inhibitors, was found by Roughton & Booth (97, 99) to be 9×10^{-3}. Using a highly purified preparation from horse erythrocytes, Kiese (100) obtained values of 1.2×10^{-3} at pH 7.4 and 2.2×10^{-3} at pH 9.3. The affinity of the enzyme for sulfanilamide appears, therefore, to be at least one thousand times greater than its affinity for carbon dioxide. According to Kreps (101), one mole of carbonic anhydrase in diluted human blood can hydrate at least 8×10^{5} moles of carbon dioxide per minute at 0°C. The role of carbonic anhydrase in the parietal cells of the gastric mucosa was discussed briefly by Bull & Gray (102) in relation to theoretical aspects of the anionic exchange which takes place in the mucosal cells during the formation of hydrochloric acid. Factors affecting the activity and stability of carbonic anhydrase were studied by Bakker (103), by Leiner (104), and by van Goor (105). As a measure of activity of the enzyme, Mitchell et al. (106) have recommended the use of the difference between the unimolecular constants of the catalyzed and uncatalyzed reactions. This difference is a linear function of the concentration of enzyme expressed in terms of volume of liquid or weight of solid preparation used.

In confirmation of previous work (107) the administration of sulfanilamide to domestic fowl was found to be associated with marked defects in the calcification of the eggshell (108, 109, 110). While it has not been proved that these defects are attributable to an inhibition, *in vivo*, of the activity of the carbonic anhydrase of the shell gland by sulfanilamide or by a metabolic derivative of sulfanilamide, it is interesting to note that there appears to be a direct correlation between the carbonic anhydrase activity of the gland, on the one hand, and productivity and shell-thickness, on the other (108). Bernard & Genest (109, 110) observed that the administration of sulfapyridine also caused a diminution in shell-thickness and they attributed the effect to an inhibition of some other enzyme. Other substituted sulfonamides such as sulfathiazole, sulfaguanidine, and sulfadiazine had a negligible effect, whereas the effects of certain sulfonamides in which the $-SO_2NH_2$ group was unsubstituted were similar to those of sulfanilamide.

Carboxylases.—The rate of decarboxylation of pyruvate by yeast carboxylase was found by Wallerstein & Stern (111) to be depressed by bisulfite when the latter was present in stoichiometric ex-

cess over the pyruvate or in equimolecular amounts at low substrate concentrations. The inhibitory effect appeared to be the result of the formation of a pyruvate-bisulfite complex which is less readily attacked by carboxylase than is free pyruvate, presumably because the enzyme has a lower affinity for the complex than for pyruvate. Extracts of carp spleen were also inhibitory (112); since they contained the thiamine-destroying Chastek paralysis factor it is possible that some destruction of cocarboxylase may have occurred, though, as the authors stated, there is as yet no proof of this assumption.

Sevag *et al.* (113) have shown that sulfonamides inhibit the activity of the carboxylases of yeast and bacteria, and that sulfathiazole, in comparison with other sulfonamides which are less closely related structurally to diphosphothiamine, causes a considerably greater inhibition of bacterial decarboxylase. More recently (114) it was observed that the inhibition of bacterial and yeast carboxylases by sulfathiazole can be partially reversed by diphosphothiamine, indicating competition between the two compounds for the enzymes. Para-aminobenzoic acid, which inhibits only slightly the carboxylase of *E. coli,* can reverse the stronger inhibition caused by sulfathiazole but not that caused by acetaldehyde (115).

Parvé (116) made a comparative study of the carboxylase activity of untreated yeast and that of a synthetic enzyme prepared by adding magnesium and cocarboxylase to apocarboxylase. The amount of coenzyme required to produce a definite activity in the synthetic enzyme was much greater than the amount associated with the same activity in the untreated yeast. The optimum pH of the native enzyme was somewhat lower than that of the synthetic enzyme. The latter value, pH 6.2, is believed to be the resultant of two different processes having different pH-optima, namely, the synthesis of the holoenzyme, a process whose optimum pH is 6.8, and the decarboxylation of pyruvic acid, having an optimum pH of 5.6.

An enzyme catalyzing the reversible transformation of oxalosuccinic acid into α-ketoglutaric acid and carbon dioxide was identified by Ochoa & Weisz-Tabori (117) in extracts of pig heart. The decarboxylation of oxalosuccinic acid was accelerated by manganese ions but was not appreciably influenced by magnesium ions present in the same molar concentration; oxaloacetic acid was not decarboxylated by the enzyme.

Amino acid decarboxylases.—Gale, Epps & Taylor have obtained cell-free preparations of six bacterial decarboxylases which act spe-

cifically on *l*-lysine (118), *l*-tyrosine (119), *l*-histidine (120), *l*-glutamic acid (121), *l*-ornithine (121), and *l*-arginine (121). Although the histidine and glutamic acid enzymes resisted dissociation into coenzyme and apoenzyme, the activity of each of the other four preparations was shown to depend on the presence of a codecarboxylase which could be separated from the enzyme protein. Decarboxylase activity was restored by adding to the apoenzymes boiled extracts of a wide variety of biological materials, including animal and plant tissues, bacteria, and yeasts (122). From dried brewers' yeast a concentrated preparation having a coenzyme activity some 15,000 times greater than that of the starting material was obtained. The purified coenzyme contained carbon, hydrogen, and nitrogen but, apparently, no phosphorus or sulfur. Studies (121) of the influence of inhibitors on the activity of the six cell-free decarboxylase preparations revealed a much more marked sensitivity to inhibition by iron and by hydrazine on the part of the four dissociable enzymes, in comparison with those in which it was not possible to demonstrate the presence of a coenzyme.

A close relationship between the vitamins of the B_6 group and the amino acid decarboxylase activity of bacteria has been established in investigations at Cornell and at Cambridge. The observation of Gale (123) that strains of *Streptococcus fecalis* which normally possess tyrosine decarboxylase activity do not contain the enzyme when grown in simplified media was followed by the demonstration by Bellamy & Gunsalus (124) that the active enzyme is formed in appreciable quantities only if the culture medium contains amounts of nicotinic acid and of pyridoxine in excess of growth requirements. Later, the same workers (125) observed that *S. fecalis* grown in pyridoxine-deficient media, though almost inactive towards tyrosine, can be reactivated by adding pyridoxal to the cell suspension; the dried cells, on the other hand, were activated (126) by the simultaneous addition of pyridoxal and ATP but not by pyridoxal alone. Umbreit & Gunsalus (127) prepared cell-free extracts of *Escherichia coli* which when tested at pH 5.0 were shown to contain the glutamic acid enzyme, although, at this pH, activity towards tyrosine, lysine, arginine, and histidine was completely lacking. In contrast with the glutamic acid decarboxylase of *Cl. welchii* which Gale found to be incapable of resolution, the corresponding enzyme of *E. coli* was partially resolved by dialysis of the extract or by incubating it for several days at 0°C. and at pH 7.0, the criterion of resolution being the reduced

activity of the preparation as compared with its activity after the addition of coenzyme. Activity was restored by adding the "natural" codecarboxylase prepared from yeast according to the method of Gale & Epps (122) or a synthetic product made by treating pyridoxal with phosphorylating agents (128) or pyridoxal and ATP. Similar results were obtained (128) in investigations of the tyrosine decarboxylase activity of cell-free extracts of *S. fecalis*. The activity of preparations of the partially resolved arginine decarboxylase of *E. coli* was shown by Umbreit & Gunsalus (127) to be augmented by the addition of synthetic coenzyme prepared by phosphorylating pyridoxal and by the natural codecarboxylase prepared from yeast; but the addition of pyridoxal plus ATP was without effect, presumably because of the lack of the necessary auxiliary enzymes. Activation of the tyrosine apoenzyme by a heat-inactivated preparation of arginine decarboxylase pointed to a close similarity between the two coenzymes, if, indeed, they are not identical substances. Gale & Epps (122) had similarly shown that tyrosine apodecarboxylase can be activated by a boiled preparation of lysine decarboxylase, and had concluded that the two coenzymes are identical. The suggestion of the Cornell workers that pyridoxal phosphate may constitute a general coenzyme of amino acid decarboxylases is supported by the observations of Baddiley & Gale (129). They compared the effects of an excess of an impure preparation of phosphorylated pyridoxal in restoring the activity of the specific apodecarboxylases for tyrosine, lysine, ornithine, and arginine, with the effects of an excess of purified codecarboxylase prepared from yeast. The two preparations, when present in excess, were equally effective in activating each of the four apoenzymes though the degree of activation varied among the four systems. Like the natural coenzyme, the synthetic preparation was fairly stable in hot alkaline solution, but the coenzyme activity of both preparations was readily destroyed by heating in acid solution. Further analytical studies revealed the presence of 1 per cent of phosphorous in the natural (yeast) coenzyme which was previously stated (122) to be phosphorus-free. The Cambridge workers also observed that pyridoxal itself exercised codecarboxylase activity only when ATP was present. According to Cohen & Lichstein (130), deficiency of pyridoxine in the medium in which *S. lactis* R is cultured is associated with a marked reduction in the tyrosine decarboxylase activity of this organism. Further evidence that codecarboxylase is a derivative of pyridoxine was obtained by Bellamy *et al.*

(131) who showed that each of the three compounds, pyridoxine, pyridoxal and pyridoxamine, gives rise to codecarboxylase in growing cultures of *Streptococcus faecalis* R, *Neurospora sitophila*, and *Saccharomyces carlsbergensis*. Non-proliferating cells of *S. fecalis* were unable to effect the synthesis of the coenzyme from pyridoxamine except when pyruvic acid was also present. As the authors suggest, it is probable that pyridoxal is first formed by transfer of the amino group of pyridoxamine to pyruvic acid and that the pyridoxal is then phosphorylated to form the coenzyme.

From the evidence briefly summarized above it is almost certain that pyridoxal phosphate is the coenzyme of amino acid decarboxylases. It may not be unfair to add that final proof will be established when chemically pure pyridoxal phosphate is isolated from the coenzyme fraction and is shown to possess the power of activating the several apoenzymes.

The possibility of using amino acid decarboxylases in the estimation of amino acids and in the analysis of proteins has been explored with considerable success. Following the suggestion of Gale & Epps (132) that the specific *l*-lysine decarboxylase of *Bacterium cadaveris* might be so employed, Zittle & Eldred (133) devised a convenient manometric method for the determination of *l*-lysine in protein hydrolysates. Neuberger & Sanger (134) used a similar enzyme, extracted from the same organism, in a study of the formation and disappearance of lysine in tissue slices. Gale (135) has used the specific decarboxylases in determining the amounts of lysine, tyrosine, glutamic acid, ornithine, and histidine in synthetic mixtures and in a number of proteins. The enzymic method is more rapid and more convenient than some of the methods previously used and it yields results which are in close agreement with those reported in the literature.

A discussion of recent work on the amino acid decarboxylases of mammalian tissues is included in a review by Blaschko (136). In another paper, Blaschko (137) reports that the manometric measurement of the *l*-cysteic acid decarboxylase activity of extracts of the liver of thiamine-deficient rats indicates a marked decrease in activity associated with the vitamin deficiency; he attributes the reduced activity to restricted intake of food rather than to any direct effect of thiamine-deficiency. In contrast with the observation of Medes & Floyd (138) that rat kidney contains an enzyme which removes the carboxyl group from cysteic acid without affecting the amino group,

Blaschko could find no evidence of the decarboxylation of cysteic acid by rat kidney. He had previously obtained similar results with the kidney of the dog, the cat, and man (139).

The anaerobic decarboxylation of 3,4-dihydroxyphenylalanine (*l*-dopa) by extracts of tissues of the rat, the guinea pig, the rabbit, the monkey, and man was investigated by Page (140). Enzyme activity was expressed in terms of the amount of hydroxytyramine formed, as determined by its effect on the blood pressure of cats. Decarboxylase activity was detected in all species except the rat which, it is said, is nevertheless one of the few animals in which a marked rise in blood pressure follows the injection of *l*-dopa. Of nine human kidneys obtained at autopsy, eight to thirty-six hours after death, only two showed any activity. In one fatal case of eclampsia the kidneys, removed four hours after death, possessed no dopa-decarboxylase activity. A similar result was obtained with normal and eclamptic human placentas. The author concluded that dopa-decarboxylase is not responsible for the hypertension associated with eclampsia. D. E. Green is reported to have demonstrated that the activity of dopa-decarboxylase depends on a coenzyme similar to that of purified transaminase (147). According to Bellamy *et al.* (131) the code-carboxylase content of rat muscle and liver, when tested as an activator of the tyrosine decarboxylase apoenzyme of *Streptococcus faecalis,* is directly related to the pyridoxine content of the diet.

Transaminase.—Proof of the existence of the glutamate-aspartate transaminase in various bacteria, including bacilli, staphylococci, streptococci, pneumococci, and nitrogen-fixing organisms, was established by Lichstein & Cohen (141). The activity of the enzyme in the bacteria was as great as, or even greater than, that of animal tissues, when compared on the basis of total nitrogen content. In the case of the bacterial studies, in which cell suspensions were used, it was necessary to limit the duration of the transamination reaction to a period of five minutes, in order to prevent the rapid destruction of the resultant aspartate by accompanying enzymes.

The possibility that pyridoxal and pyridoxamine may be interconvertible in transamination reactions was suggested by Snell (142) on the basis of some experiments on the growth of yeast and bacteria. Subsequent chemical studies (143) indicated that pyridoxal reacts reversibly with glutamic acid at elevated temperatures to form pyridoxamine and α-ketoglutaric acid, and that similar reactions appear to occur between pyridoxal and other amino acids and between pyri-

doxamine and other α-ketoacids. That pyridoxine or a closely related derivative may be associated with transamination in biological systems was indicated by further observations of Schlenk & Snell (144): (*a*) that transaminase concentrates prepared from pig heart contain appreciable amounts of "total vitamin B_6" as determined by a yeast growth method; (*b*) that the transaminase activity of extracts of tissues of B_6-deficient rats is consistently lower than that of extracts of the corresponding tissues of normal animals; (*c*) that the transaminase activity of deficient tissues was, in some experiments, augmented by the addition of pyridoxal or of pyridoxamine, provided that ATP was also added. Schlenk & Fisher (145) achieved considerable purification of the glutamate-aspartate transaminase of pig heart and observed that as the activity of the preparations increased so also did their vitamin B_6-content. It was suggested that a pyridoxine derivative serves as a rather firmly bound prosthetic group. Two distinct transaminating enzymes, namely the glutamate-aspartate and the glutamate-alanine enzymes, were prepared in purified form from pig heart by Leloir & Green (146). They reported that they could find no evidence of a dissociable prosthetic group or of significant amounts of B-complex vitamins in either enzyme, but it was stated (145) that on re-examination of the glutamate-aspartate transaminase Green was able to demonstrate the presence of a pyridoxine derivative [see also (147)].

The glutamate-aspartate and glutamate-alanine transaminase systems of *Streptococcus faecalis* R were studied by Lichstein *et al.* (147). By varying the composition of the synthetic culture medium it was possible to modify greatly the transaminase activity of the organisms. When pyridoxal was present in the medium in a concentration of 30 μg. per cent, the harvested cells were found to possess high transaminase activity; but if alanine were substituted for pyridoxal, the cells were either devoid of activity or the activity was greatly reduced. Dried preparations and cell-free extracts of organisms grown in pyridoxal-free media evidently contained transaminase apoenzymes; for the addition of either pyridoxal phosphate or, in some cases, of pyridoxal plus ATP stimulated the transaminase activity of these preparations to levels comparable with the activity of organisms grown in media containing excess pyridoxal. These results support the view that pyridoxal phosphate functions as a coenzyme in transamination and, considered in conjunction with the role which pyridoxal phosphate appears to play in the bacterial de-

carboxylation of amino acids, they point to an intimate association between pyridoxine and its derivatives on the one hand and the metabolism of amino acids in bacteria, on the other. Though it is still too early to reach any definite conclusion regarding the coenzyme function of pyridoxine and its derivatives in animal tissues, the limited evidence so far obtained is consistent with the view that these substances may function in the animal organism in a manner similar to that in which they appear to function in the bacterial cell. Further investigation is needed, however, in order to establish with greater certainty the role of the compounds in the metabolism of amino acids in animal tissues.

Amylases.—Weill & Caldwell (148, 149) believe that free sulfhydryl groups are essential components of β-amylase of barley and barley malt. They base this conclusion on the results of studies of the inhibitory effects of various reagents on the activity of highly purified preparations of the enzyme. Treatment with nitrous acid caused a diminution in activity which was less than that suffered by pancreatic amylase under similar conditions. Kinetic studies indicated that the sharp initial loss of activity caused by nitrous acid was not due to destruction of essential amino or tyrosine groups; after inactivation of this kind, full activity could be restored by treatment with hydrogen sulfide, indicating that essential sulfhydryl groups were oxidized during the treatment with nitrous acid. More prolonged treatment with this acid caused irreversible inactivation which was attributed mainly to a reaction with tyrosine groups rather than with free amino groups. Studies of the effects of other reagents which are believed to be specific for sulfhydryl groups supported the view that these groups are essential for activity.

The secretion of amylase into the culture fluid by *Clostridium acetobutylicum* was demonstrated several years ago by Johnston (150) who purified the enzyme to a considerable degree and described a number of its properties. Hockenhull & Herbert (151) have prepared this amylase in a concentrated form containing only about 5 per cent maltase and they have extended our knowledge of its mode of action. Its formation appears to depend on the presence of starch in the culture medium, and it is said to convert starch completely to maltose. In this latter respect it differs from other known amylases.

A number of other papers on amylases have appeared, but only very brief reference to a few of these can be made. Caldwell *et al.* described a procedure yielding highly active preparations of the amyl-

ase of *Aspergillus oryzae* (152). *Rhizopus japonicus* (*Amylomyces-β*) contains both α- and β-amylases: in the early stages of growth the mold secretes mainly α-amylase; later, β-amylase is formed and it reaches its maximum at the time of spore formation (153). Beckord *et al.* investigated the production of amylase by bacteria of the *B. sub-tilis* group when grown under different conditions on a medium consisting of autoclaved wheat bran moistened with phosphate buffer (154). The complementary action of *Aspergillus oryzae* in the hydrolysis of raw starch by the α-amylase of pancreas is attributed by Schwimmer (155) to the influence of maltase which is present in the mold. Olson *et al.* described methods for the determination of α- and β-amylases in malt (156). Kneen made a comparative study of the development of amylases in germinating cereals (157). He also studied the amylase system of sorghum malt and found it to be composed of α-amylase accompanied by relatively minute quantities of β-amylase (158). Davidson investigated the total and free amylase content of dormant cereal seeds and the influence of a variety of substances on the diastatic power of flour and of malt (159).

Lipase and phospholipase.—On examination of the products of hydrolysis of olive oil by pancreatic lipase *in vitro* and by the enzymes functioning in the small intestine of the rat, Frazer & Sammons (160) demonstrated in both cases the presence of partial hydrolytic products in the form of substances having a high acetyl value. During the first five hours of hydrolysis *in vitro* no free glycerol could be identified in the mixture but the acetyl value of the glyceride fraction rose twelve-fold; the presence of mono- and di-glycerides was demonstrated by isolating them as the di-phthalate and mono-phthalate, respectively. The acetyl value of the glyceride fraction of the intestinal contents of rats sixteen hours after intragastric administration of olive oil indicated the presence of about 30 per cent monoglyceride in this fraction. These experiments support the view that complete hydrolysis of fat is not necessary before absorption can take place and they form the basis of the authors' suggestions that one of the functions of lipolysis is the formation of two of the components of an effective emulsifying system composed of monoglyceride, fatty acid, and bile salt, and that the lower glycerides constitute a nucleus for the synthesis of phospholipids at the oil-water interface in the intestinal cell.

Gomori (161) has described a method of demonstrating the sites of lipase activity in paraffin sections of acetone-fixed tissues. The

insoluble calcium salt of palmitic or stearic acid is formed as the result of hydrolysis of a water-soluble ester of the acid in the presence of calcium chloride. The calcium salt of the fatty acid is then transformed to the lead salt and by subsequent blackening with hydrogen sulfide the site of lipolysis is identified.

The name "phospholipase" has been adopted by Fairbairn (162), in preference to the more specific name "lecithinase" to designate an enzyme, in the venom of the cottonmouth moccasin, which liberates unsaturated fatty acids from phospholipids prepared from beef brain, with the formation of lysophospholipids. Lecithins and cephalins are hydrolyzed by the enzyme whereas cerebrosides, sphingomyelins, lysophospholipids, and acetal phospholipids are resistant to its action.

Fairbairn (163) believes that methods frequently used in extracting lipids from tissues are such as to favor the liberation of relatively large amounts of free fatty acids from the tissue phospholipids by enzymatic hydrolysis. When the methods of extraction were modified in such a manner as to reduce to a minimum the activity of the constituent phospholipases, Fairbairn found that the maximum average amounts of free fatty acids in the extracts were much smaller than many values previously reported.

Schmidt et al. (164) observed that although the incubation of phosphatides with preparations of intestinal mucosa liberated nearly all the phosphorus in inorganic form, similar incubation with preparations of gastric mucosa and of pancreas led to the formation of an acid-soluble compound which could be rapidly hydrolyzed in acid solution, yielding glycerophosphate and choline. Since the compound reacted with periodate in equivalent proportions and was levorotatory it is believed to be (-)α-glycerylphosphorylcholine.

Cholesterol esterase.—Pantaléon and co-workers (165 to 169) have investigated the synthetic and hydrolytic action of this enzyme. When pancreatic fistula juice was added to heated blood serum containing bile salts, a rapid conversion of free cholesterol to cholesterol esters took place at pH 7.5 to 8.3. In addition to cholesterol esterase, pancreatic fistula juice contains a lecithinase which liberates fatty acids from phospholipids and is distinguishable (167) from the lipase also present by the fact that the lecithinase, in contrast with lipase, is slightly inhibited by bile salts and is strongly inhibited by phlorhizin and by monobromoacetic acid. The synthesis of cholesterol esters in blood serum is attributed (168, 169) to a coupled action of serum lecithinase and cholesterol esterase; the first enzyme liberates fatty

acids from serum phospholipids and the second one catalyzes the union of fatty acid with cholesterol.

Cholinesterases.—Sufficient experimental evidence has now been accumulated to justify the conclusion that the animal body contains cholinesterases of more than one type, having different properties and exhibiting different activities and specificities. Some of this evidence has been considered in previous reviews (170 to 173), and therefore, in any further discussion of the differentiation of the cholinesterases, it is necessary to refer only to more recent contributions having a bearing on this problem. While it is true that the specific and non-specific cholinesterases exhibit a consistent difference with respect to their activity-substrate concentration relationships when studied under the conditions originally adopted (174), Mendel & Rudney (172) have stated that this difference must be regarded as a secondary criterion of distinction. Specificity of action is, in their opinion, the only true criterion of differentiation: the specific or "true" cholinesterase hydrolyzes only choline esters, whereas the non-specific or "pseudo" cholinesterase can hydrolyze a variety of esters including those of choline. Augustinsson (175) has stated that the cholinesterase of horse serum, which is mainly non-specific in its activity, and that of red cells, which is specific, differ greatly in their rates of migration in an electric field and exhibit different susceptibilities to inactivation in acid solution. Langemann (176) observed that the non-specific cholinesterase is only slightly influenced by caffeine when the latter is present in concentrations which greatly inhibit the specific esterase. In a study of the action of several drugs on cholinesterase, Nachmansohn & Schneemann (177) noted that only caffeine and theobromine were exclusively inhibitory toward the specific enzyme. As a means of distinguishing cholinesterase from "unspecified" esterases which also hydrolyze acetylcholine, Rothenberg (178) suggested the use of the substrate phenylacetylcholine which, though completely resistant to cholinesterase, was hydrolyzed by other esterases present in animal tissues and in blood serum. Hawkins & Gunter (179) observed that a prostigmine analogue,[1] in low concentrations, has a much greater inhibitory action on the non-specific than on the specific cholinesterase. It will be recalled that Mendel *et al.* (180) found that benzoylcholine, though resistant to the action of the specific esterase, is readily hydrolyzed by pseudo-cholinesterase;

[1] Hoffmann-La Roche Nu- 683.

both enzymes hydrolyze acetylcholine but only the specific esterase hydrolyzes acetyl-β-methylcholine. Sawyer (181) has recently discovered in the liver of the rabbit and the guinea pig an enzyme which hydrolyzes benzoylcholine but not acetylcholine, and which differs, therefore, from pseudo-cholinesterase. He suggests the name benzoylcholinesterase to emphasize the distinction and he sounds a note of caution as to the acceptance of benzoylcholine hydrolysis as an absolute measure of pseudo-cholinesterase activity.

Nachmansohn & Rothenberg (182) concluded that the concentration of acetylcholine associated with maximum activity of the specific cholinesterase is at a much higher level than that previously reported by Mendel & Rudney (174). It now appears, from a study by the latter workers (183), that the discrepancy can be largely, if not entirely, attributed to a difference in the salt concentration of the enzyme-substrate mixtures used in the two laboratories. Those used by Nachmansohn & Rothenberg contained magnesium chloride and relatively large amounts of sodium chloride in addition to $0.025\ M$ sodium bicarbonate, whereas the mixtures of Mendel and co-workers contained only sodium bicarbonate, in the same concentration. On gradually increasing the salt concentration by adding increasing amounts of potassium chloride it was shown (183) that the optimal concentration of acetylcholine gradually changed from the low value, previously reported, to a value approaching that of Nachmansohn & Rothenberg (182). Furthermore, the activity of the enzyme, in mixtures containing acetylcholine in a concentration which is optimal in the absence of added potassium chloride, was reduced in the presence of this salt.

Cholinesterase in homogenized nerve tissue of different kinds was said by Nachmansohn & Rothenberg (182) to exhibit a "relative specificity" for acetylcholine. By this they meant that, of several choline esters investigated, none was hydrolyzed by the nerve enzymes at a higher rate than acetylcholine. The experimental data indicated that under the conditions adopted, all substrates being present in the same initial concentration $(0.015\ M)$, the hydrolysis of acetylcholine was faster than that of any other ester of choline, with the possible exception of propionylcholine which was hydrolyzed by one preparation somewhat more rapidly than was acetylcholine. In no case except that of acetylcholine, was any information given regarding the concentration of substrate necessary to saturate the enzyme or regarding the inhibitory effect, if any, of excess substrate (an effect

which was very apparent in the case of acetylcholine) ; it is not inconceivable that the adoption of some other initial substrate-concentration and of other experimental conditions, in comparing the activity of the enzymes on the several esters, might have revealed a quite different relationship among the substrates, with respect to their susceptibilities to hydrolysis *in vitro*. The conclusion that "the main feature of cholinesterase is the fact that no other substrate is split at a higher rate than acetylcholine" would seem, on the basis of the limited evidence presented, to be unjustified.

Richards & Cutkomp (184) observed that, in contrast with the nerve enzymes studied by Nachmansohn & Rothenberg (182), those of bees and cockroaches were nearly three times as active against acetyl-β-methylcholine as against acetylcholine. But in view of the differences in the composition of the mixtures employed by the two groups of workers it is difficult to be certain that the difference in the ratio of the rates of hydrolysis of the two substrates by the cholinesterase in vertebrate and in invertebrate nervous tissue represents a true difference between the enzymes, as suggested.

The suggestion of Mendel & Rudney (174) that it is the specific, rather than the non-specific, cholinesterase which acts *in vivo* to bring about the rapid hydrolysis of acetylcholine is supported by several observations. For example, the non-specific enzyme is absent from brain tissue of vertebrate animals (185) ; it is not present in the blood and tissues of the ox and the sheep (186) ; it is still present, though in reduced amount, in the superior cervical sympathetic ganglion of the cat at a time when the specific esterase has completely disappeared, following the complete degeneration of the preganglionic nerve endings after preganglionic section (187). Hawkins & Gunter (179) observed that when the prostigmine analogue, previously mentioned, was injected into animals in amounts sufficient to cause almost complete inhibition of the non-specific cholinesterase in the blood and tissues, it did not elicit any symptoms which could be related to the accumulation of acetylcholine. These symptoms appeared only when sufficient inhibitor was administered to cause marked inhibition of the specific cholinesterase also.

The distribution of cholinesterases in human tissues was investigated by Langemann (176). Skeletal muscle, thymus of very young infants, brain, and hypophysis contained only the specific enzyme and the ovary only the non-specific esterase ; the thymus of older children and the heart contained both enzymes. Unlike vertebrate

brain which contains only specific cholinesterase activity (185), the peripheral nerves of the rat and the dog contain both enzymes (188). The differentiation of the nervous system in the developing embryo of the spotted salamander was found to be accompanied by an increase in the concentration of cholinesterase in the nervous tissues; more-over, neural structures secondarily induced by experimentally im-planted tissue resembled primary nervous tissue with respect to cho-linesterase activity (189). The cholinesterase of human and dog erythrocytes is said to be attached to the stroma of the cells (190).

Only the briefest reference can be made to a number of other interesting observations. Serum cholinesterase reaches a low level after prolonged administration of barbiturates (191), and, in epilep-tic patients after sudden withdrawal of the drug, Schütz (192) ob-served a sharp rise in the number of seizures while the enzyme was still at a low level. The seizures became less frequent again when the serum cholinesterase started to rise and usually disappeared when the enzyme reached its highest level. In the guinea pig, prolonged treatment with barbiturates did not cause any significant change in the cholinesterase activity of brain tissue though that of the spinal cord, muscle, and serum was considerably reduced (193). Muscular dystrophy in rats, induced by denervation or by vitamin E deficiency, was accompanied by a marked reduction in cholinesterase in the gas-trocnemius muscle (194). Greatly reduced activity of the serum enzyme of guinea pigs was associated with severe deficiency of vita-min C (195), and in thiamine-deficient animals the activity of cho-linesterase in the sciatic nerve was reduced (196). Traumatic shock caused a rapid fall in the serum enzyme, both in anesthetized and in unanesthetized animals (197). The serum cholinesterase of guinea pigs was not significantly altered in histamine shock although the activity of the enzyme in vitro was partially inhibited by histamine (198). Frommel et al. (199) made an extensive study of the influence of ions on the activity of horse serum cholinesterase. Though the activity of cholinesterase has been said to depend upon the presence of free sulfhydryl groups, the activity of the cholinesterases in serum and in brain homogenate was unaffected by oxygen at a pressure of seven atmospheres (200). In a report of investigations of the toxic properties of certain compounds in relation to their value as agents in chemical warfare, Bodansky (201) referred to a compound, the nature of which was not disclosed, which had the effect of reducing to almost negligible values the cholinesterase activity of the serum

of human subjects who were exposed to the agent, without eliciting any symptoms of very serious distress. In view of the fact that the cholinesterase in human serum is mainly non-specific in activity, this observation would seem to support the view that the function of the non-specific cholinesterase in the hydrolysis of acetylcholine *in vivo* is not an important one. No information was given regarding the effect of exposure to the compound on the specific cholinesterase.

The mode of action of acetylcholine in the central nervous system forms the subject of a comprehensive review by Feldberg (202). He has included a summary of present knowledge of the enzymatic synthesis of acetylcholine in the animal organism.

Crystalline enzymes.—Reference has already been made to recent reports of the crystallization of mexicain (3), of rennin (34), of phosphorylase-*b* of muscle (70), and of phosphopyruvate transphosphorylase (92). To these may be added fumarase (203), the alkaline phosphatase of kidney (204), and the oxidizing enzyme, *d*-glyceraldehyde monophosphate dehydrogenase of muscle (205).

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PLANT CARBOHYDRATES

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The reserve carbohydrates most widely distributed in the plant kingdom are sucrose and starch, and these also constitute the principal sources of carbohydrate food for man and the animals.

Sucrose appears to function in the plant either as a temporary reserve (chiefly in the leaves) or as one of the forms in which carbohydrate is translocated. Starch, on the other hand, represents a more permanent form of storage and is found as such in seeds and in roots and tubers. The synthesis of these carbohydrate reserves in plants and their subsequent breakdown at will is a subject to which increasing attention has been paid in recent years, with the result that the outlines, at least, of the metabolic picture are visible.

STARCH

Constitution

The most significant advance towards a clarification of the chemistry of starch has been the belated recognition that most natural starches are composed of at least two chemically different molecular species. For these, the terminology of Maquenne is appropriated and the name "amylose" used for the linear unbranched component and "amylopectin" for the branched-chain component of starch. The developments of this conception up to the year 1943 have been carefully examined by Hassid in Volume XIII of the *Annual Review of Biochemistry,* where the evidence for the chemical heterogeneity of starch is presented.

Much of the confusion and apparent contradiction in the earlier literature on starch is due in a large measure to the failure to recognise this heterogeneity and much of the work there detailed will need to be repeated on the separated components.

Separation of the components.—Of all the methods that have been proposed in the past for the fractionation of starch, only three achieve the separation of the branched and unbranched components. Two of these methods originated with Tanret (1), namely, (*a*) absorption of the amylose fraction on cellulose, and (*b*) the leaching out of the

amylose component by water at a temperature below the gelatinisation point of the starch. The first method has been used by Pacsu (2) and by Kerr (3); the second was developed by Baldwin (4) and extensively applied by Meyer (5). It appears likely that the electrophoretic method of Samec (6) also effects a true separation. The discovery by Schoch (7) that amylose was selectively precipitated when a starch paste was autoclaved with butanol led to a method of separation which has been widely used. The same worker was later able to show that the linear component of corn starch adsorbed fatty acid and thereby became insoluble in water. Thus, amylose was separated in 29 per cent yield from a paste of defatted corn starch when the autoclaved solution was treated with oleic acid (8). Alternative precipitants have been described by Whistler & Hilbert (9) and by Haworth, Peat & Sagrott (10). The first-named authors found that amylose formed a molecular complex with nitropropane which was water-insoluble, and they were thereby able to achieve a separation at least as good as that obtained by the butanol method in respect of amylose purity. The method of Haworth, Peat & Sagrott has some technical advantages over earlier methods and the separation of the starch components is more clear-cut. The precipitant is thymol which is added to a cold dispersion of starch in water. The precipitated fraction of the starch has a higher amylose content, as measured by the iodine-staining method of McCready & Hassid (11), than the fraction precipitated by butanol from the same starch.

By a combination of the water-extraction and the butanol-precipitation methods, Kerr & Severson (3) were able to separate the amylose fraction of corn starch in a micro-crystalline form. It has been shown by Bear (12, 13) that the crystallinity is different if branched-chain instead of normal alcohols are used as precipitants. Other modifications of the butanol method have been used by Whistler & Hilbert (14), Pigman (15), and Boise & Vallières (16). The last-named workers use a dialysed alkaline dispersion of starch which is treated at 37° C. with n-butanol and the amylose so precipitated is described as being crystalline.

The branched component of starch either does not form a complex with such amylose-precipitants as butanol or thymol or, if it does, the complex is soluble in water. The amylopectin, being a residue in the mother liquor after the amylose has been precipitated, is usually not obtainable in as pure a form as the amylose component. It is known that the amylopectin prepared by either the butanol or thymol

methods always contains some of the linear component. An amylo-
pectin has, however, been obtained from potato starch which appears
to be substantially free from amylose, inasmuch as the colour of its
stain with iodine contains no blue component. The iodine complex is
reddish-brown in colour and is indistinguishable from that given by
glycogen. Amylopectins of this degree of purity are prepared by pre-
cipitation of the amylose component with a mixture of thymol and
cyclohexanol (or methylcyclohexanol) (10).

There are unusual features in this separation of branched- and
unbranched-chain polymers and not the least unusual is the variety of
substances which will function selectively as amylose-precipitants.
These include butyl and amyl alcohols (but not ethyl or methyl alco-
hols) ; certain phenols (but not phenol itself) ; terpene derivatives such
as menthol and borneol; nitroparaffins, amyl acetate, methyl ethyl
ketone (but not acetone), pyridine, butyric acid, oleic acid, and others
(7 to 10, 13, 17). Whistler (9) attempts to correlate the amylose-
precipitating power of substances with their ability to form hydrogen
bonds with the polysaccharide hydroxyl groups. Such a correlation
if established would not explain, however, why butanol selectively
precipitates amylose and methanol makes no such selection, nor yet
why amylopection remains unaffected by these reagents.

The conformation of the amylose molecule.—Some insight may be
gained into this puzzling precipitation effect by a consideration of the
molecular conformation of amylose in its water-soluble and water-
insoluble forms and of the related phenomena of retrogradation.

With respect to the insoluble forms of starch much information is
derived from a study of x-ray diffraction patterns made by French,
Rundle, and their co-workers.

In 1941, Bear & French (18) concluded that the diffractions of
various starch grains were those of a single component of the starch ;
that there was a ring-for-ring correspondence between the A- and B-
type patterns [Katz nomenclature (19)] ; and that, moreover, in-
soluble retrograded whole starch showed the same pattern as the
grains of potato starch (B-type) while A-patterns were obtained with
starches "crystallised" from concentrated solutions at higher tem-
peratures. Rundle, Daasch & French (20) confirmed these observa-
tions and took an important step forward by demonstrating that the
diffraction pattern of a film of retrograded amylose, prepared by
evaporation of a concentrated solution, gave the same B-pattern as
the powder photographs of retrograded whole starch or of natural

starch granules. Measurement of the dimensions of the unit cell indi-
cated that it contained eight glucose residues. The conclusion was
drawn that the B-pattern was characteristic of retrograded amylose
which existed in the solid form as an extended chain, comparable with
that of cellulose or chitin.

Katz (19) had shown that starch which was precipitated from
aqueous solutions by alcohol addition gave a different type of pattern—
the "V"-pattern. The V-pattern is given by butanol-precipitated
amylose with such sharpness that Rundle & Edwards (21) were able
to calculate the cellular dimensions and to show that butanol-amylose,
which usually is easily dispersible in cold water, exists not in the ex-
tended form of insoluble, or retrograded, amylose, but is coiled in a
tight helix, each turn of which consists of six glucose residues. Mean-
while, Rundle & French (22) had shown that dry butanol-amylose
absorbed iodine vapour whereas amylopectin did not. An x-ray dia-
gram of the iodine-amylose complex indicated that the amylose re-
tained its helical structure and that in all probability the iodine mole-
cules were accommodated within the spiral, with such packing that
there was one iodine molecule in each turn of the helix. Amylopectin,
from which the last traces of amylose are removed by adsorption on
cellulose, gives an amorphous diffraction diagram (3) as does gly-
cogen, and it must be concluded that the "A," "B," and "V"-patterns
of whole starches are due entirely to the amylose component. In A-
and B-starches the amylose exists as a linear extended chain and is
insoluble in water; in V-pattern starches the amylose chain is coiled
into a helix, alternate helices being laid down parallel to each other
but oriented in opposite directions (21).

Although V-pattern amylose dissolves in water and the extended
chain amylose (B-pattern) is insoluble, the x-ray evidence says noth-
ing about the conformation of the amylose chains when in solution.
We do know, however, that amylose may be obtained from aqueous
solutions in the form either of an extended chain (retrograded amyl-
ose) or of a helix (butanol-amylose). The retrograded amylose can
not be directly redissolved in water, whereas the helical form dissolves
readily if the precipitant in the complex is removed. Retrograded
amylose bears a close resemblance to cellulose. Each is insoluble in
water, probably for the same reason, namely, because of hydrogen
bonding between the hydroxyl groups of adjacent linear chains (23).
This correspondence between the linear molecules of amylose and cel-
lulose is even more clearly demonstrated by the properties of their

derivatives. Amylose triacetate is fibrous and indistinguishable in appearance from cellulose acetate. It forms tough films which, when stretched, give x-ray fibre patterns showing a high degree of crystallinity. Moreover, the mechanical properties of the two substances are remarkably similar (24, 25). It would seem, therefore, that in the solid acetate amylose is in the extended form. It is probably linear also in the methyl ether, even in solution, since Haworth, Heath & Peat (43) found an equivalence of molecular weight of a methylated synthetic amylose as determined by end-group assay and by viscosity measurement: an equivalence that was obtained only if Staudinger's constant for methyl cellulose (an extended chain) was used in the viscosity calculation. An equivalence was also observed by Meyer *et al.* (27) in the molecular weight of potato amylose as determined by end-group assay and by osmotic pressure measurement, a demonstration that this amylose was essentially an unbranched structure. The linearity of amylose chains is also supported by the viscosity measurements made by Foster & Hixon (28) on solutions in ethylene diamine. A wide variation in the chain-lengths of amyloses of different origins was revealed by this work. Thus, potato amylose showed a degree of polymerisation of 500 glucose residues whereas synthetic amylose, at the other end of the scale, had only 85 units (see ref. 26, for end-group assay). Amylose, water-extracted from maize, had a chain-length of 115 glucose units but the amylose precipitated from maize starch by butanol had the much longer chain of 250 units. It would appear from the last observation that a considerable heterogeneity with respect to length of chain is to be expected of natural amyloses. Clearly in the case of corn amylose, the shorter chains are more readily soluble in water than the longer chains.

The question as to whether starch is composed of only two molecular species or whether amylose and amylopectin represent merely the extremes of a series in which the length of the unit chain progressively increases and at the same time the extent of branching decreases in passing from amylopectin to amylose, has not yet been answered with any certainty. Rundle and his co-workers see in the very different light absorption capacities of the iodine complexes of amylose and amylopectin (29) and in the different type of reaction each undergoes with iodine, evidence in favour of the theory of discontinuity of structure between amylose and amylopectin (30). On the other hand Kerr, who also uses a spectrophotometric method for the estimation of amylose (31), inclines to the opposite view and has very recently

(23) produced further supporting evidence based, like the work of Foster & Hixon (28), on a study of the viscosities in ethylene diamine solution of the fractions and subfractions of various natural starches. Kerr concludes that all the components of starch are relatively asymmetric in shape. This would mean, for instance, that the component referred to as amylose is not homogeneous but is a mixture containing branched and unbranched molecules. It is understood, of course, that the average length of unit chains is much greater, and the degree of branching much smaller, in the amylose fraction than they are in the amylopectin fraction.

Estimation.—To conclude this section mention will be made of methods that are at present employed for determining the relative proportions of amylose and amylopectin in starches. Apart from the method of weighing the separated components, they are all based upon complex formation with iodine and include the potentiometric method of Bates, French & Rundle (30), the absorptiometer method of Mc-Cready & Hassid (11), and the spectrophotometric methods of Kerr & Trubell (31) and Baldwin, Bear & Rundle (29).

The Action of Amylases

It is natural that one of the oldest enzymic processes used by man should have been the subject of wide and intensive study. The results of such study could not lead, however, to a consistent theory of amylase action so long as whole starch was held to be a homogeneous substrate. The heterogeneity of starch having now been demonstrated and its components made available for separate investigation, the situation is favourable for a thorough-going re-examination of the whole subject. Some results of this reinvestigation have already been published and will be briefly discussed here.

The newer results have amply confirmed the view that the amylases may be divided into at least two classes—the α- and β-amylases—which differ profoundly in their manner of catalysing the hydrolytic fission of glycosidic linkings in starch. The mode of action of β-amylase, the "amylase of dormancy," is relatively simple and consists of the progressive removal of maltose units from the nonreducing end of a chain of glucose residues mutually linked by α-1 : 4-links. The action of the α-type of amylase, the "amylase of activity," is more complex, its main features comprising the production, in the early stages of reaction, of shorter chain dextrins with the liberation of little or no reducing sugar. In the later stages of reaction when the

dextrins themselves are undergoing hydrolysis, maltose makes its appearance.

The unbranched component of starch is completely hydrolysed by β-amylase, the sole product of hydrolysis being maltose (32 to 35), whereas amylopectin, either natural or synthetic, is converted to maltose to the extent of only about 50 per cent (36, 37), the residue being a limit dextrin (dextrin-A, α-amylodextrin, erythrogranulose) which is not further attacked by β-amylase.

The incompleteness of the hydrolysis of amylopectin by β-amylase is ascribed to the presence of the cross linkages in the amylopectin. The action of β-amylase on the two components of starch is essentially the same in each case, namely, an attack directed at the nonreducing end of a chain of glucose residues. The unbranched amylose offers no obstruction to this attack and is consequently converted completely to maltose. The hydrolysis of amylopectin begins at the branch terminations and proceeds along the branches until the bifurcations are reached, when the action of β-amylase ceases and dextrin-A results. Diagrams illustrating this process are given by Meyer & Bernfeld (36) and by Haworth, Kitchen & Peat (37). The first-named authors represent amylopectin by a structure with multiple branching, but the latter consider that all the known facts are adequately covered by a simpler laminated structure with single instead of multiple branchings and that there is no immediate necessity to postulate a more complicated and less well-ordered pattern. In the laminated structure, the cross-links between unit chains engage the reducing end of one chain and a centrally placed glucose residue in the adjacent chain.

Dextrin-A is a limit dextrin so far as the action of β-amylase is concerned, but it may be isomerised in such a way that it once more becomes a substrate for the further action of β-amylase. This isomerisation or "sensitisation" is accomplished in a remarkable manner, namely, by the incubation of dextrin-A for a brief period (a few minutes only) with salivary amylase. Haworth, Kitchen & Peat (37) suggested in explanation that saliva might contain an enzyme system which catalyses not only the hydrolytic fission of the 1 : 6-glycosidic cross-linkages in dextrin-A but also their resynthesis to give "sensitised" dextrin-A. Meyer & Bernfeld (36) found that the prolonged action of yeast maltase on dextrin-A (dextrin I, in the nomenclature of these authors) produced a similar effect inasmuch as the dextrin II which resulted was susceptible to attack by β-amylase. It is clear,

however, that limit dextrin II cannot be identical with "sensitised" dextrin-A since the saliva used in the formation of the latter was known to be free from maltase or other α-glucosidase.

Salivary amylase is usually classed as an α-amylase and it is well known that α-amylases rapidly degrade starch (and therefore amylopectin) far beyond the stage represented by dextrin-A. In other words, the α-type of amylase includes among its functions that of catalysing the cleavage of α-1 : 6-links. Whether the resynthesis inferred by Haworth, Kitchen & Peat is also a function common to all α-amylases remains to be seen, but the recent isolation from the potato of an amylolytic enzyme which also possesses synthesising powers suggests that this might be so (35, 38, 39).

Myrbäck (26) favours the view that amylopectin is constituted of a main chain of glucose members from which side chains branch at intervals. In principle, this formulation is the same as that proposed by Staudinger (52) in 1937. According to Myrbäck & Sillén (51), the facts, qualitative and quantitative, of amylolysis are satisfactorily interpreted in terms of such a formulation.

THE SYNTHESIS OF AMYLOSE AND AMYLOPECTIN

The extensive researches of Cori and his associates on glycogen metabolism, culminating in the isolation from muscle of a crystalline enzyme which catalysed the reversible conversion in *vitro* of glucose-1-phosphate into amylose (40), were paralleled by the isolation by Hanes (32, 41) of a phosphorylase from the pea and the potato which catalysed the same reversible reaction in plants. Here at last was concrete evidence that the biological synthesis of reserve polysaccharides in both plants and animals might be explicable otherwise than by the unsatisfactory hypothesis that synthesis is merely a reversed amylolysis, for which hypothesis there is no real evidence at all.

Two curious facts emerge from this work. Firstly, the crystalline phosphorylase when isolated from muscle effects the synthesis of a blue-staining amylose with an average chain-length of 200 glucose members (42) whereas the phosphorylase *in vivo* invariably synthesises the highly ramified structure of glycogen. Secondly, although the product of the action *in vitro* of purified potato phosphorylase on glucose-1-phosphate is again an amylose [chain-length 80 to 90 glucose units (43)], the starch synthesised in the plant contains only 25 per cent of this component. It has thus been clearly demonstrated that the synthetic action of phosphorylase, using glucose-1-phosphate

as substrate, must be supplemented, in living tissue, by some other factor (or factors) which is capable of introducing cross-linkages be-tween the linear chains.

An enzyme with this property has been shown by Cori & Cori (44) to be present in animal tissues. Thus, when an extract of liver or of heart which had, *per se,* no phosphorylase activity, was added to a digest of crystalline muscle phosphorylase with glucose-1-phosphate, the product was not an amylose but instead a red-staining polysac-charide which appeared to be glycogen. In the same paper, the authors indicated that two synthesising enzymes probably existed also in the grain of waxy maize inasmuch as a phosphorylase-contain-ing extract from the grain synthesised amylose only, and yet the starch in this plant appears to consist entirely of amylopectin.

The isolation from potato of a "cross-linking" enzyme which, in conjunction with potato phosphorylase, catalyses the synthesis of amylopectin from glucose-1-phosphate was briefly announced in a communication by Haworth, Peat & Bourne (39) in 1944 and more fully reported later by Bourne & Peat (38) and Bourne, Macey & Peat (35). The cross-linking enzyme is referred to as Q-enzyme, and potato phosphorylase when purified by Hanes' method as P-enzyme. A combination of P- and Q-enzymes acting upon glucose-1-phosphate yielded a polyglucose which, in respect to the colour it gives with iodine its mode of hydrolysis with β-amylase, its solubility in water, and the stability of the solution (no retrogradation), could not be dis-tinguished from "cyclohexanol"-amylopectin. A provisional end-group assay showed the repeating chain unit to contain about 20 glucose members. Osmotic pressure measurement of the molecular weight of the methylated synthetic amylopectin indicated the presence of six of these repeating chain units (45). It will be seen that the amylose and the amylopectin synthesised respectively by P-enzyme alone and by P- and Q-enzymes acting together have smaller molecular weights than the corresponding components of natural potato starch.

Q-enzyme performs a double function. Not only is it instrumental in the formation of 1:6-linkages, but it also catalyses the scission of 1:4-links. In fine, it is an amylase but an amylase whose mode of action distinguishes it from amylases of either of the known α- or β-types (35). In its presence, amylose is rapidly hydrolysed but the hydrolysis is not accompanied by the appearance of reducing sugars. In this respect the action of Q-enzyme resembles the initial stages of the hydrolysis of amylose by an α-amylase. Nevertheless, with Q-prep-

arations which have been freed as far as possible from the ordinary amylases of the potato, the later stage of α-amylolysis, marked by the liberation of reducing sugar, is not observed. Briefly, Q-enzyme converts a nonreducing and blue-staining amylose into a nonreducing, red-staining polyglucose which Bourne & Peat (38) suggest might be an amylopectin. This reaction cannot be a phosphorolysis for the reason that it takes place in the absence of phosphate ion. Further evidence that Q-enzyme is identical with neither the α-amylase of saliva nor the β-amylase of soybean comes from a comparison of the relative stabilities of the three enzymes towards heat treatment and towards precipitation with alcohol.

With regard to the power that salivary amylase appears to possess of synthesising 1:6-cross linkages in the sensitisation of dextrin-A (37), it is conceivable that the α-amylase of saliva is associated with an enzyme of the Q-type. The experiments described do not exclude such a possibility but they do seem to suggest that the Q-enzyme of potato may exert its distinctive function without being associated with a true α-amylase. In this connection it is interesting to recall the observation of Cori & Cori (44) that salivary amylase does not implement the synthesis of glycogen by muscle phosphorylase in the way that liver extract does. This observation might mean that saliva does not, in fact, contain an enzyme of the Q-type, i.e., an enzyme specifically catalysing the synthesis of 1:6-links.

The synthesis and degradation of whole starch in the plant by the agency of P- and Q-enzymes is tentatively pictured by Bourne & Peat (38) in the following manner.

Glucose-1-phosphate
↑↓   P-enzyme

[Pseudo amylose]
(single chains of
ca. 20 glucose units)

Q-enzyme   Q-enzyme   P-enzyme

Amylopectin   Amylose
(branched structure; unit chain of   (unbranched chain of > 80
20 glucose residues)   glucose units)
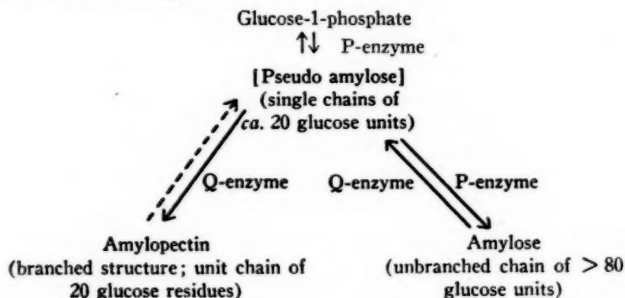
Polysaccharide catalysts in the synthetic process.—Evidence is accumulating that a purified phosphorylase (P-enzyme) cannot initiate

synthesis with pure glucose-1-phosphate as substrate unless there are present also catalytic amounts of a polyglucose. Thus, Cori & Cori (46) found that the incubation of glucose-1-phosphate with highly purified muscle phosphorylase was ineffective in amylose synthesis until a small amount of glycogen was added. Other muscle preparations, while not completely inactive, showed a more or less prolonged retardation of synthesis. This initial lag was also abolished when glycogen was added. Plant phosphorylases also need polysaccharide "starters," as was demonstrated in the first place by Hanes (32) and thereafter by Green & Stumpf (47). The latter authors, studying the effect of a number of carbohydrate "starters" on P-enzyme from potato, showed that amylose synthesis proceeded only if whole starch, glycogen, or a dextrin produced from whole starch was present. Glucose, sucrose, maltose, and the Schardinger dextrins did not function as activators. More significantly, synthetic amylose failed to catalyse its own synthesis. This observation was confirmed by Hassid, Cori & McCready (42) by the demonstration that synthetic amylose does not activate crystalline muscle phosphorylase.

These experiments strongly suggest that before P-enzyme can function as a synthesising enzyme it must be activated by the presence of a trace of a branched-chain polyglucose which may be either amylopectin or glycogen, or dextrins derived by hydrolysis from either of these.

Hidy & Day (48) expressed the view that the inability of amylose to function as a primer may be due to its sparing solubility in water and proceeded to show that hydrolytic degradation products of amylose were markedly more effective than amylose itself. Nevertheless, it was also shown that maximal enzyme activation was attained much more rapidly with branched-chain dextrins than with the unbranched-chain dextrins from amylose.

Sumner, Somers & Sisler (49) do not agree that amylose is ineffective as a primer in synthesis by the phosphorylase of potato or jack bean. Unfortunately this conclusion is based upon the use of an amylose, separated from corn starch, which would almost certainly contain some amylopectin. It is conceivable therefore that the activation observed was due to this amylopectin.

Weibull & Tiselius (50), extending the work of Green & Stumpf (47) on the activating power of dextrins derived from starch, fractionated, by adsorption, two limit dextrin mixtures prepared by Myrbäck. The first of these limit dextrins [presumably an abnormal

α-dextrin, in Myrbäck's classification (51)] proved to consist for the main part of trisaccharides which were ineffective as primers in amylose synthesis. On the other hand, the second limit dextrin, which was stated to be a normal α-dextrin in the sense that its constituents were unbranched structures and contained only α-1 : 4-glucosidic linkages, did function as a primer, as did each of its constituent tri-, tetra-, penta-, and hexa-saccharides. The authors conclude that the minimum catalytic element essential for the amylose synthesis is a chain of three glucose units combined by α-1 : 4-glucosidic links, a conclusion which accords with the views of Sumner and of Hidy & Day but not with those of the other workers mentioned above.

It is obviously too early at present to speculate on the nature of this priming mechanism, nevertheless, hypotheses are not lacking. Sumner *et al.* (49), for instance, finding that the presence of a small amount of achroodextrin catalyses the synthesis of amylose, whereas with larger amounts of dextrin a red-staining polysaccharide results, suggest that P-enzyme requires a polysaccharide "pattern" or nucleus, the chain (or chains) of which it proceeds to lengthen by the apposition of glucose units derived from glucose-1-phosphate. If very few molecules of the pattern are present, comparatively few long amylose chains are synthesised. If however, many molecules of the "pattern" are available, the chains synthesised are intermediate in length but are formed in greater number. Kuzin & Ivanov (53) decided, however, that the nature of the product depends on the kind of polysaccharide catalyst used. In the presence of starch, potato phosphorylase synthesised sparingly-soluble, blue-staining amylose, whereas, when glycogen was the "primer," the product was water-soluble and gave a reddish colour with iodine.

Bliss (54) reports that the nature of the product synthesised by the phosphorylase of waxy maize depends not so much upon the presence of a "primer" but rather upon the conditions of digestion. Incubation for a short time at room temperature yields amylose, but at a higher temperature the same digest apparently yields amylopectin. It seems desirable that details of this work be published.

SUCROSE

The metabolic history of sucrose is in many respects the story of starch all over again. It is well known that in plant economy sucrose and starch are interchangeable assets and it is not surprising to find that modern research is revealing more and more the essential same-

ness in Nature's methods of ensuring an adequate reserve of carbohydrate.

As with starch, it was the breakdown rather than the synthesis of sucrose that first received attention and the hydrolytic enzyme, invertase, was shown to have an almost universal distribution. The mechanism of sucrose formation, however, remained obscure until very recently. It was inevitable that sucrose synthesis should come to be regarded as a reversed inversion and indeed some workers have produced evidence purporting to demonstrate that invertase catalyses synthesis as well as hydrolysis (55, 56). Other investigators have, however, failed to substantiate this claim (57, 58).

Oparin & Kursanov (55) stressed the important role of inorganic phosphate in the alleged synthesis by invertase and many suggestions have been made that sugar phosphates play a part in the synthesis of sucrose. Thus, Burkard & Neuberg (59) showed that although beets with high sucrose content contain only traces of organic phosphorus, the leaves of the sugar beet contain appreciable amounts of hexose phosphates. Kriukova (60) concluded that phosphorylation was essential for sucrose synthesis inasmuch as iodoacetate which inhibits phosphorylation also inhibits sucrose synthesis in plants. Similar views were expressed by Hassid (61), Kursanov (56), and Hartt (62). The last-named author showed that pure glucose was absorbed from solution by the roots of the sugar-cane plant and transformed into sucrose. He also showed that phosphate ion was vitally necessary in this transformation and was of the opinion that fructose diphosphate was formed as an intermediate.

This hypothesis has not been supported as yet by the isolation from a plant source of the enzymes responsible for sucrose synthesis, but Doudoroff, Kaplan & Hassid (63) have succeeded in preparing such an enzyme from a bacterial source. The dried cells of *Pseudomonas saccharophila* contain an enzyme system which catalyses the breakdown of sucrose in the presence of phosphate ion with the formation of glucose-1-phosphate and fructose. The glucose phosphate isolated was undoubtedly α-glucose-1-phosphate inasmuch as it functioned as substrate for the synthesis of amylose by potato phosphorylase. Furthermore, the sucrose phosphorylase catalysed the reverse reaction, i.e., the synthesis of sucrose from glucose-1-phosphate and fructose. The dried bacteria contained invertase and it appeared that the phosphorolytic and hydrolytic enzymes were competing for the sucrose. In a later paper, however, Hassid, Doudoroff & Barker

(64) describe the use of an enzyme preparation which was freed from invertase by the method of Doudoroff (65). By the action of this preparation on a mixture of the potassium salt of glucose-1-phosphate and fructose at pH 6.8, there was formed a crystalline nonreducing disaccharide which was indistinguishable from natural cane-sugar. It was thus that the first laboratory synthesis of sucrose was achieved. In the past many attempts to effect the chemical synthesis of sucrose have been made but they have been made in vain. The fact that there are four different ways in which glucopyranose and fructofuranose may be linked glycosidically is probably responsible for this lack of success. The synthesis achieved by Hassid *et al.* sheds some light on the nature of the glycosidic linkage in sucrose. The glucose-1-phosphate employed in the synthesis of starch and also of sucrose is the α-form. In the starch synthesis the glycosidic links formed are α-links and it is reasonable to suppose, therefore, that glucose exists also in the α-form in sucrose.

It is worthy of note that the fructose supplied in this synthesis is in the pyranose form. In the course of the reaction, ring change must occur since the ketose constituent of sucrose is fructofuranose. Hassid (61) had previously assumed that fructose had to be phosphorylated in the sixth position before synthesis of sucrose could take place. It has now been recognised that this assumption is unnecessary and that the ring change to fructofuranose may be effected either by the sucrose-phosphorylase itself or by an accompanying enzyme in the bacterial preparation. The mechanism of this ring change does not involve phosphorylation of fructose in the sixth position inasmuch as Doudoroff (65) has shown that fructofuranose-6-phosphate cannot be substituted for fructopyranose in the synthesis of sucrose.

The phosphorylase of *Pseudomonas saccharophila* is without action on trehalose, maltose, raffinose, glycogen, or starch nor is any synthetic action observed if glucose-1-phosphate is replaced by other phosphoric esters of aldoses or ketoses, including maltose-1-phosphate (63 to 67). The enzyme is evidently highly specific, at least so far as the phosphorylated aldose component of the substrate is concerned. Such high specificity is not, however, observed with respect to the ketose component. Doudoroff, Hassid & Barker (67) have been able to prepare two analogues of sucrose by the action of the bacterial phosphorylase on a mixture of glucose-1-phosphate with, respectively, *l*-sorbose and *d*-ketoxylose. No reaction was observed with a mixture of glucose-1-phosphate and either *d*-tagatose or *d*-xylose. These au-

thors with Dore (68) have more fully investigated the nonreducing disaccharide produced from glucose-1-phosphate and *l*-sorbose and have shown that it is easily hydrolysed by acids, that the sorbose is in the furanose form, and that the glucose and the sorbose are each in the α-configuration. The polysaccharide is thus probably correctly described as α-*d*-glucopyranosido-α-*l*-sorbofuranoside. This isomer of sucrose has not hitherto been detected in any living organism.

DEXTRANS AND LEVANS

Starch and sucrose are not the only forms in which glucose is stored by plants nor is sucrose the only reserve carbohydrate containing fructose. In some species, the favoured form of storage is as the polyfructofuranose, inulin. In dahlia roots and in artichokes no starch is associated with the inulin but in certain plants, such as the snowdrop bulb (69), starch and inulin are formed side by side. It is difficult to avoid the impression that the biological synthesis of starch, sucrose, and inulin must be closely related processes. This we know to be true in the case of starch and sucrose but the biological aspect of inulin chemistry has, up to the present, been completely neglected. However, other types of polyfructofuranoses such as the levans occur naturally and these are becoming increasingly the subject of biochemical investigation. Levans, in which fructofuranose units are linked by 2:6-linkages, occur in the higher plants, e.g., in *Poa trivialis* (70), but are more usually encountered as products of the action of microorganisms (such as, for example, *B. mesentericus*) on sucrose solutions.

The dextrans (polyglucoses in which the units are linked by 1:6-glucosidic linkages) may be classed with the levans since both are characteristically products of the activity of certain microorganisms which metabolise sucrose. Dextrans, like levans, have been shown to be produced also by certain of the higher plants, e.g., in barley roots (71), but it is doubtful if either plays an essential part in the general carbohydrate metabolism of plants.

The enzyme system (levansucrase) responsible for the sucrose-levan conversion in *Aerobacter levanicum* has been isolated from the organism by Hestrin & Avineri-Shapiro (72) who show that it catalyses the following transformation:

$$(n + m) \text{ sucrose} + (n)H_2O \rightarrow (n + m) \text{ glucose}$$
$$+ (n) \text{ fructose} + [\text{levan}]$$
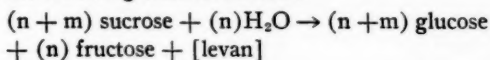
Raffinose acts similarly as a substrate.

The production of glucose in this reaction ranks equally with levan formation as a product of levansucrase action. It remains to be ascertained whether fructose production is also a function of the same enzyme. In the analogous case of dextran production from sucrose by cell-free extracts of *Leuconostoc mesenterioides* (73) simple inversion of sucrose does not occur, the reaction proceeding according to the following equation:

$$(m) \text{ sucrose} \rightarrow (m) \text{ fructose} + [\text{dextran}]$$
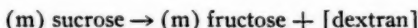
Neither of these reactions appears to be reversible and for neither is phosphate ion required.

Hestrin (72) draws attention to the striking analogy between the production of polysaccharide from sucrose and from glucose-1-phosphate. This analogy finds expression chiefly in the absence of any energetic coupling of synthesis with sugar desmolysis, and in the fact that the substrate in both cases is not a simple hexose but a glycosidic derivative higher in energy content than a simple hexose.

The enzymic transformations described in this review are conveniently summarised by being fitted into the single scheme illustrated below. This diagram is a modification of that given by Avineri-Shapiro & Hestrin (74), who propose the generic name polymerosynthease to describe "the enzyme entities which mediate the polymeric steps of the scheme."

$$H_3PO_4$$
$$+$$
Glucose + Levan $\leftarrow$ Sucrose $\rightarrow$ Dextran + Fructose

$$H_3PO_4 + \text{Glycogen} \rightleftarrows \alpha\text{-Glucose-1-phosphate} \rightleftarrows \begin{cases} \text{Amylose} \\ + \\ \text{Amylopectin} \end{cases} + H_3PO_4$$
$$+$$
$$\text{Fructose}$$
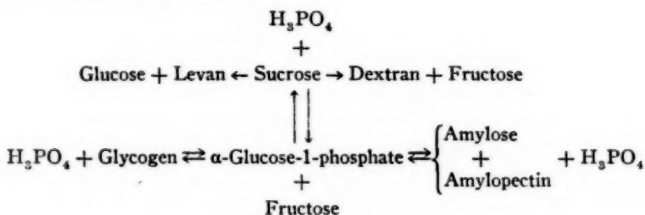
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THE CHEMISTRY OF THE LIPIDS

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This review will follow the general outline used by the writer two years ago (1). No attempt will be made to include work of the past year on the chemistry of such lipids as the antioxidants and the sterols; of the latter group, however, reference is made to cholesterol as a constituent of the tissue lipids.

During the past year a treatise on "Industrial Oil and Fat Products" by Bailey (2) has been published. An especially interesting review of the lipoproteins by Chargaff (3) describes methods of preparation, types of linkage between lipid and protein, and properties of the lipoproteins. McNair (4) in an article mainly of biological interest has discussed plant fats in relation to environment and evolution. The intermediary metabolism of the fatty acids has been reviewed by Stadie (5). The reader is again referred to the very complete annual review on fats and oils by Piskur (6) in *Oil and Soap,* the official Journal of the American Oil Chemists Society.

ANALYTICAL METHODS

No new methods for the analysis of fatty acid mixtures have been proposed. However, improvements on and new applications of previously reported procedures have been numerous.

The ester fractionation method for the separation of fatty acid mixtures with the use of highly efficient columns is now being used in most laboratories, although it is perhaps fair to say that no two laboratories employ exactly the same distillation set-up. An interesting discussion of the theory and practice of such distillations by Norris & Terry (7) is accompanied by data showing the results of typical distillations with the use of the Pennsylvania State College (8) and Podbielniak (9) columns. Starting with a mixture of equal parts of methyl stearate and methyl oleate, and using the Podbielniak column, they obtained 95 per cent methyl oleate in the first fractions. However, no separation was obtained when a comparable mixture of the

* The writer is indebted to Mrs. Betty Orians and to Miss Mary Frechtling for their aid in collecting the material for this review.

oleate and linoleate was distilled. It seems unlikely to them that unsaturated esters of the same carbon series can be separated by this method because of possible association of these esters. Another new, commercially available distillation apparatus has been described by Todd (10). It is adaptable to quantities of esters varying from 2 to 5,000 cc., and the columns have efficiencies of 30 to 50 theoretical plates. Generally speaking, it would seem to be undesirable in the case of most ester mixtures to use charges of over 1,000 cc., due to the increased likelihood of pyrolysis of the more unsaturated esters with long exposure to the conditions of the distillations.

Baldwin & Longenecker (11) tested the accuracy of the ester distillation method on two known mixtures of pure methyl esters. Using only 40 to 45 gm. of charge, they obtained the following results on one of their mixtures, known values in weight percentage being given in parenthesis: laurate 6.4 (6.1); myristate 15.1 (14.3); palmitate 20.6 (21.0); stearate 11.8 (11.5); oleate 29.2 (29.8); linoleate 8.8 (9.2); and linolenate 8.1 (8.1). Linoleate and linolenate were determined spectrophotometrically in several fractions. These are remarkable results in view of the small specimen employed, and illustrate the accuracy of the method as carried out in their laboratory. They emphasize the importance of the application of other methods of identification, such as crystallization, bromination, etc., in the elucidation of the composition of such mixtures. Jack & Henderson (12) described in detail the ester fractionation method as applied to the analysis of the component acids of several glyceride fractions of butter fat, including a description of their apparatus, which is of the type employed by Longenecker and his associates.

Few important contributions in the field of chromatography are noted. Applications of this method to the chemistry of drying oils are reviewed by Mills (13) and by Fisk (14). Walker (15) separated the glycerides of linseed oil by passing a solution of 2.5 to 3.0 gm. of the oil in 50 cc. of n-hexane over a column of alumina at the rate of 200 cc. per hr. and developing the chromatogram with 300 cc. more of the solvent. Four zones representing glycerides with 7, 6, 5, and 4 double bonds were noted, the more unsaturated glycerides being found at the top of the column. By repeated fractionation linoleyl-dilinolenin was obtained, along with evidence for the presence of trilinolenin. In the course of comparing alumina, silica gel, and carbon as adsorbents, Kaufmann & Wolf (16) found that tributyrin was more strongly adsorbed by the first two substances and that it could be separated

from mixtures with tristearin. The separation of the *cis-trans* isomers, oleic and elaidic, and erucic and brassidic acids, was accomplished in part by the use of silica with benzol or petroleum ether as solvents. Kiselev *et al.* (17) studied the effect of the temperature of dehydration of silica gel on its absorptive capacity for acetic, propionic, and stearic acids, and showed that adsorption decreases with rise in temperature of dehydration. Thus silica gel dehydrated at 485° adsorbed nearly twice as much stearic acid as when dehydrated at 800°. Chromatographic separation of the noncholesterol unsaponifiable fractions of human cancerous and noncancerous livers was carried out by Stanger and co-workers (18). After removing the cholesterol and passing the unsaponifiable portion in petroleum ether over alumina, four fractions were obtained. From the second of these fractions pure hepene octahydrochloride, $C_{45}H_{76} \cdot 8HCl$, was isolated. The other fractions were not identified.

The spectrophotometric method for the identification and estimation of dienoic, trienoic, and tetrenoic acids in fatty acid mixtures continues to be the major interest among analytical procedures, and several important refinements and applications have been described. Descriptive accounts of this subject have been written by Kass (19), Brode (20), and Barnes (21). A recording quartz spectrophotometer has been described by Beckman (22). Mellon (23), in one of a series of articles included in a symposium on spectrochemical methods, reviewed the general methods and applications of light absorption spectrometry, and included a great deal of valuable information for those who contemplate working in this field.

Most workers who are employing this method for the first time are authenticating their results by complete descriptions of their procedure and by the use of data obtained by them on pure compounds. The reference values for the specific absorption coefficients at various wave lengths of linoleic, linolenic, and arachidonic acids, after alkali isomerization to conjugated diene, triene, and tetraene systems, were provided by Beadle & Kraybill (24) and were also included in the *Annual Review of Biochemistry* for 1944 (25). Some variations from these values are being reported. Baldwin & Daubert (26) described the absorption curves of alkali isomerized synthetic monolinolein and monolinolenin as well as the corresponding triglycerides of these acids and found that their data could be applied accurately in the estimation of these acids in the natural glycerides. Their values of $E_{1cm}^{1\%}$ for the isomerized pure methyl esters are slightly lower than the values re-

ported by Mitchell, Kraybill & Zscheile (27) : for linoleate, 802 at 234 mμ; for linolenate, 529 at 234 mμ and 453 at 270 mμ. In the course of an attempt to analyze cholesteryl linoleate by this method Front & Daubert (28) found that this wax is only partly saponified during alkali isomerization; hence the method gives erratic results. A modified procedure, which is described, gives complete saponification and isomerization and yields satisfactory results in determining the purity of the ester.

Brice & Swain (29) in attempting to apply the methods previously mentioned (24, 27) to materials having very low proportions of nonconjugated polyethenoic fatty acids encountered a number of difficulties: the development of a strong and variable ultraviolet absorption on heating in ethylene glycol with potassium hydroxide in air; relatively high background absorption by extraneous compounds; and, in mixtures in which the ratio of conjugated (diene) to nonconjugated compounds is high, the question as to what happens to the conjugated constituents during the isomerization treatment. Their modified method comprises measurement of the absorption of the sample before and after isomerization; the use of a more suitable solvent, glycerol; correction of the data for extraneous absorption; correction of the data for conjugated constituents originally present and remaining undestroyed by the isomerization; and calculation of the proportions of conjugated and nonconjugated diene, triene, and tetraene fatty acid constituents in the sample. Data are included showing absorption curves of various oils, pure esters, and acids before and after isomerization, analysis of a tallow by their procedure in comparison with results by other proposed methods, and a comparison of results with and without the several corrections proposed by them. In addition to tallows and specimens of perilla and soybean oils, they report analyses of several mixtures of known composition with satisfactory results. Their data, together with several previous and concurrent investigations, show the presence of naturally occurring conjugated fatty acids in fats and oils and in the pure acids made from these fats. For example, one of the tallows examined was found to contain 0.9 per cent conjugated diene, 0.14 per cent triene, and 0.03 per cent tetraene, an over-all content of over 1 per cent of conjugated material. The linoleic, linolenic, and arachidonic acid contents by the proposed method were 3.39, 0.61, and 0.68 per cent respectively. Thus, approximately one-fourth of the dienoic acid originally present in this tallow was conjugated dienoic acid. It should be further pointed out that these

authors name the nonconjugated dienoic, trienoic, and tetrenoic acids found in this tallow specifically as linoleic, etc., on the assumption that these are the only specific acids of these types present. This criticism will be amplified later. The procedure devised by Brice & Swain has been applied to a more extended series of fatty materials by Brice *et al.* (30). The results of analyses of lard, additional specimens of tallow, perilla and tobacco seed oils, and other products of economic importance have been compared with those obtained by the thiocyanometric calculations. The value of the spectrophotometric method in the detection of small amounts of conjugated compounds, originally present in these fats, is again apparent, since no other method (probably including the diene number determination) will detect these conjugated substances satisfactorily. In discussing the accuracy of the procedure the authors point out that many factors contribute to the uncertainty of results, especially the possibility of the natural occurrence of isomers having different rates of isomerization from those shown by the standards employed (v.i. Lemon). Incidentally, the arachidonic acid contents of lards found by this method agree well with the values reported by Brown & Deck (31) who based their values on estimates from the yield of octabromides.

Beadle, Kraybill & Stricker (32) were able to distinguish between lard and vegetable shortening, because of the fact that the former contains small amounts of arachidonic acid, which becomes spectroscopically visible after isomerization, while vegetable fats are practically free of tetraenoic acids. Mixtures containing up to 50 per cent lard could be detected qualitatively. Similarly, O'Connor *et al.* (33) were able to detect soybean oil in cottonseed and peanut oils because of the presence of linolenic acid in the soybean oil and its absence in the others. Hilditch, Morton & Riley (34) have reported a detailed description of their experiences in connection with developing the spectrographic method for determination of linoleic, linolenic, and eleostearic acids in oils containing these acids. Because linoleic acid isomerizes to conjugated diene forms relatively slowly, the maximum value for $E_{1cm}^{1\%}$ at 234 mμ being reached only after 60 minutes heating at 180°, and because maximum values for linolenic acid are attained at 180° in ten minutes (decrease thereafter due to probable thermal polymerization), they prefer to employ two temperatures and two times of isomerization: for linolenic acid at 268 mμ, 170° and 15 minutes; for linoleic acid, at 234 mμ, 180° and 60 minutes. When both acids are present, the increment of $E_{1cm}^{1\%}$ at 234 mμ due to conjugated dienes pro-

duced from linolenic acid is allowed for by employing the $E_{1cm}^{1\%}$ value of 569 at 234 mμ for linolenic acid under these conditions. In oils containing eleostearic acid, this acid is first determined from the extinction coefficient of the fatty acids at 268 mμ without isomerization. The amount of eleostearic acid found must be taken into account in the calculations for linoleic and linolenic acids after isomerization. The extinction coefficients of pure α-eleostearic acid were determined in several solvents by O'Connor et al. (35) and the values used in the determination of this acid in tung oil. A comprehensive tabulation of spectroscopic data on fats, fatty acids, and their esters was published by Rusoff et al. (36). The data include a bibliography, solvents used, wave lengths of maxima, and the approximate numerical values of the absorption coefficients when known.

The value of the spectrophotometric method in the examination of the fatty acids is thus being more and more proved as new adaptations of the method as an analytical tool appear. Not only does it permit of an accurate estimation of dienoic, trienoic, and tetrenoic acids in mixtures, but also it is now serving to direct attention to the natural occurrence of conjugated acids which may eventually prove to be significant in living processes. It would appear to be of far greater value than the diene number as a measure of conjugation, because it differentiates the several degrees of conjugation. The method has several serious disadvantages, among which are the cost of the apparatus and the availability of personnel trained to use the spectrophotometer. Another difficulty is the scarcity of pure materials for use as standards, since it seems advisable that each group check its results with pure compounds. Still a further disadvantage is the lack of specificity of the results if it is desired to state them in terms of specific acids. Thus, to state diene conjugation, after isomerization, in terms of linoleic acid is to rely on the assumption that linoleic acid is the only dienoic acid in the material under investigation. This assumption may or may not be true. In butter fat, for example, no linoleic acid is found, although isomeric linoleic acids occur in this fat to the extent of 3 to 5 per cent. In human milk fat, about half of the octadecadienoic acids is ordinary linoleic acid (37). At present practically the only way to actually identify linoleic, linolenic, and arachidonic acids is by means of their characteristic bromine addition products or the corresponding hydroxy acids. Still a final possible error in the results from the spectrophotometric method is the possibility of the presence of polyunsaturated acids which do not isomerize completely or at all to conjugated group-

ings. This possibility was pointed out in the paper of Hilditch *et al.* (34) and is borne out further by the three investigations which follow.

Lemon (38) in 1944, studying flavor reversion in hydrogenated linseed oil, found that this oil, hydrogenated to an iodine number of 120, contains about 18 per cent of an isomeric linoleic acid (octadecadienoic) which fails to isomerize with alkali. He suggested that this unusual acid was formed by preferential hydrogenation of linolenic acid whereby the 12-double bond was saturated, thus forming 9,15-octadecadienoic acid which was alkali-resistant. Daubert & Filer (39) have offered another explanation of this phenomenon, namely, that the resistant acid could have been produced by spread of the double bonds in linoleic acid, a result which would be especially important in those oils in which linoleic acid is the principal polyethenoic acid. By partially hydrogenating mixtures of purified methyl stearate and linoleate, and calculating the compostiion of the resulting mixtures from iodine numbers and spectral data, an apparent decrease in methyl stearate was noted. This erroneous conclusion is explainable if an alkali-resistant dienoic acid were formed, which at the same time gives a theoretical iodine number, but behaves as oleate in its absorption bands. Studies on pure methyl linoleate and linolenate confirmed Lemon's conclusions, although it was by no means certain that the 9,15-octadecadienoic acid postulated by Lemon is the acid in question. Daubert & Filer were able to achieve some concentration of the resistant acid by low temperature crystallization. Still further, Mattil (40) found that by heating soybean oil with nickel catalyst there was a decrease in linoleic and linolenic acids accompanied by a smaller increase in diene and triene conjugated acids. He noted especially a probable increase in isolinoleic acids. He concluded that no simple methods are available for the accurate analysis of a fat subjected to such a treatment.

A somewhat different trend in the spectrophotometric examination of fats and oils is to be found in extreme ultraviolet absorptions of the fatty acids and their compounds. Previous work by Barnes and co-workers (41) had described direct absorption of oleic, linoleic, linolenic, and arachidonic acids down to 2100 Å at which point none of the curves had reached a peak. Rusoff *et al.* (42) have continued this work in a specially devised apparatus so that readings can be taken to 1700 Å. In heptane the saturated acids have one broad band with the maximum at 2050 Å and another region of intense absorption beginning at 1850 Å and still rising steeply at 1730 Å. Absorption maxima

were observed as follows: for oleic acid at 1830 Å; for linoleic acid (by debromination) at 1900 Å; for linolenic acid (14 times recrystallized), at 1950 Å and 1750 Å; and for methyl arachidonate, maxima between 1700 Å and 2000 Å. Absorption curves for linolenic acid obtained by debromination and by crystallization were appreciably different, due possibly to the presence of *cis-trans* isomers in the former. Differences were also observed in the curves of oleic and elaidic acids and of linolenic and elaido-linolenic acids. The absorption of light by oils at very short wave lengths is directly dependent upon the fatty acid composition of the oil.

In the annual report of the Committee on Analysis of Commercial Fats and Oils (43) the hexabromide test for linolenic acid was found to be unreliable in oils containing less than 3 per cent of this acid. It seems only fair to point out that all other tests are unreliable for this purpose, unless one assumes all of the trienoic acid present in this oil to be linolenic acid. This committee also found that more accurate thiocyanogen values could be obtained when the quantity of potassium iodide used in the titration is increased from 1.00 to 1.66 gm. and that the addition of carbon tetrachloride to the reagent is undesirable. On the other hand, Lambou & Dollear (44), from a detailed study of the method, suggested the use of the following conditions: replacement of 25 per cent of the glacial acetic acid with carbon tetrachloride; the use of dry potassium iodide; the use of more potassium iodide; and meticulous care in drying glassware. Their results include thiocyanogen values of purified linoleic and linolenic acids, which in turn were analyzed spectrophotometrically.

Child (45) found that Wijs solution, kept at 81° F. for ten months, lost in titration value at the rate of about 1 per cent per month without alteration in the iodine number values obtained with this reagent. Kaufmann & Kirsch (46) found that tetranitromethane gives a red color with conjugated unsaturation in contradistinction to a yellow color with nonconjugated unsaturation. The intensity of red color is greater with triene conjugation than with diene. This reagent seems worthy of further study.

The use of low temperature crystallization in the separation of mixtures of fatty acids or of their esters is being increasingly used in the investigation of lipids. Singleton, Lambou & Bailey (47) reported a very detailed study of the separation of the solid and liquid acids of cottonseed oil. They obtained the best separations by crystallization from polar solvents, such as acetone, methyl acetate, etc. Hilditch &

Riley (48) have adapted the low temperature crystallization procedure to the separation of saturated and unsaturated acids in place of the more tedious lead salt-alcohol method. In connection with the subsequent use of ester-distillation, the method was applied successfully to the analysis of sunflower-seed, sesame, and peanut oils, the results obtained agreeing satisfactorily with analyses of these oils by the lead soap method. Somewhat higher results for linoleic acid were obtained by the crystallization method for oils rich in this acid. Somewhat better results were obtained by crystallization of the free acids than by crystallization of the methyl esters. In this work usually three or four fractions of acids were obtained by crystallization and the esters of these fractions prepared and distilled. Thus, with sunflower-seed oil, 250 gm. of acids in 1,200 cc. acetone were cooled to $-30°$, giving crystal and filtrate fractions. The former was twice crystallized from ether giving two additional filtrate fractions and a crystal fraction. These four fractions had iodine numbers of 157.9, 152.2, 123.7, and 9.2 respectively. Each was subsequently analyzed by ester-distillation. In line with their observation that more efficient procedures of crystallization could likely be found, it seems to the writer that the procedure employed by De la Mare & Shorland (49), who crystallized the methyl esters of lard from acetone (15 cc. per gm.) at $-35°$, or some modification thereof, would simplify the method so that no more than two distillations would be required. Smith & Brown (50) distilled the methyl esters of a large specimen of menhaden oil, and employed crystallization procedures to separate and study the C_{12}, C_{14}, C_{16}, and C_{18} main fractions. In a later investigation (51) they separated the esters of menhaden oil into substantially pure saturated, monethenoic, and polyethenoic fractions by crystallization methods. By distillation of the first two fractions evidence was obtained relative to the range and amounts of the carbon series in each. The polyethenoic fraction had an iodine number of 285. Pure oleic acid was isolated from this oil and evidence was presented for the presence of other octadecenoic acids. A constant temperature bath suitable for work at temperatures down to $-70°$ was described by Ruh *et al.* (52).

CHEMISTRY OF THE FATTY ACIDS

Schuette and co-workers have extended their data on solidification points of binary mixtures of fatty acids to the lower members of the homologous series, caproic and caprylic acids (53), and to the higher members, triacontanoic to tetratriacontanoic acids (54). Data on these

higher members indicate the tendency of the curves to assume a flattened form with less pronounced breaks as the homologous series of these compounds ascends. Ralston & Hoerr (55) found that the solubilities of mixtures of palmitic and stearic acids are directly dependent upon the melting point of each given mixture, the most soluble mixture being of that composition which possesses the minimum melting point of the system. Solubilization of a fatty acid is considered as a process of melting. Intermolecular association between fatty acid molecules appears to have relatively little influence in altering the characteristic solubility behavior of binary mixtures in solvents having different physiochemical properties. Solubility relations of the higher fatty acids and their esters in propane near the critical temperature were studied by Drew & Hixson (56). In the system propane-palmitic acid-stearic acid no separation of the two acids is believed possible. Dipole moments of fatty acids and glycerides in dioxane, cyclohexane, hexane, methyl cyclohexane, and benzene were determined by Stephanenko and co-workers (57, 58, 59).

In the class of new and unusual fatty acids Steger & van Loon (60) have obtained from sterculia oil a new saturated acid, sterculic acid, which adds halogens and thiocyanogen but does not hydrogenate. It yields pelargonic and azelaic acids when oxidized with permanganate. In the acids found as sterol esters in degras (wool fat), Weitkamp (61) found the normal acids from capric to hexacosanoic, two optically active 2-hydroxy acids, iso acids from C_{10} to C_{28} with the methyl group on the second carbon from the methyl end of the chain, and, in addition, a new series of acids with a methyl group on the third carbon from the methyl end of the chain (C_9 to C_{27}, and C_{31}). For these odd-carbon acids the author suggests the name anteiso acids. In addition to isolating a large series of these new acids, a method for their structural elucidation is described which is based on the number of transitions appearing in the solidification point curve of binary mixtures of the branched chain acids (or amides) with normal fatty acids (or amides). In an attempt to establish the structure of one member of the series of anteiso acids by synthesis, Velick & English (62) prepared d-14-methylpalmitic acid and found it to be identical with the corresponding acid from wool fat. Hilditch & Jasperson (63) demonstrated spectroscopically the presence of 1.5 per cent conjugated octadecadienoic acids in the C_{18} fraction of butter fat; in a concentrate of the more unsaturated acids (C_{18}) of goat milk fat they found 7.9 per cent of these unusual acids and 0.3 per cent conjugated octadeca-

trienoic acid; in the mixed acids of pasture grass both of these conjugated acids were demonstrated. Shorland (64) isolated an unusual triene acid, hexadecatrienoic acid, from rape-leaf lipids. Upon bromination it gave 72 per cent of ether-insoluble bromides of m.p. 181°. Esafov et al. (65) found a new linolenic acid (iso) in the oil of *Lappula echinata* Gilb. Its hexabromide melted at 232–34°.

Ginger & Anderson (66) isolated a series of dextrorotatory acids ($C_{24}H_{48}O_2$, $C_{25}H_{50}O_2$, $C_{26}H_{52}O_2$, and $C_{27}H_{54}O_2$) from the acetone-soluble fat of an unidentified strain of tubercle bacilli cell residues which remained from preparation of the purified tuberculin protein, PPD. Constants of the C_{26} acid agreed almost exactly with previously reported values for phthioic acid. But a mixture of the four acids would also give similar values. This raises the question of the homogeneity of phthioic acid, which was previously isolated from human tubercle bacillus, Strain H-37. By the use of an improved method of estimating terminal methyl groups, Ginger (67) showed that each of the preceding four acids contains three such terminal groups. In the wax isolated from tubercle bacilli residues from the preparation of PPD, Ginger & Anderson (68) found, in addition to the usual fatty acids, tuberculostearic acid, dextrorotatory acids similar to phthioic acid, and an *l*-rotatory acid, mycocerosic acid, $C_{30}H_{60}O_2$. The last of these is characteristic of all wax fractions of human tubercle bacilli. Polgar & Robinson (69) synthesized a number of methyl substituted acids, one of which, 3,13,19-trimethyltricosanoic acid, exhibited properties which are in agreement with those of phthioic acid.

Hydnocarpic and chaulmoogric acids were prepared (70) by distillation of the ethyl esters of the oil from *Hydnocarpus wightiana*. The absorption spectra of these acids are similar to that of ethyl-1-cyclopentene, that of chaulmoogric acid being more intense. Schuette & Roth (71) prepared pure stearic acid by a novel procedure, namely, by first isolating pure ethyl 12-hydroxystearic acid by crystallization of the hydrogenated ethyl esters of castor oil from alcohol, conversion to the free acid, preparation of 12-iodostearic acid, and reduction to stearic acid with zinc dust in glacial acetic acid. Swern et al. (72) prepared pure oleic acid (95 per cent) by low temperature crystallization of tallow fatty acids.

Several investigations have been made of the oxidation of the fatty acids and their glycerides. In one of these studies (73) methyl oleate was oxidized by passing air through the ester at 65° with cobalt oleate as catalyst. The products of the oxidation were separated into a num-

ber of fractions. Polymers, linked by oxygen, of molecular weight 1,700 were identified. A methyl ester fraction boiling between oleate and the polymers was found to be composed of esters of dimeric acids and of several isomeric monohydroxy derivatives of octadecenoic acids in which the double bond had shifted from the 9,10-position. Findley, Swern & Scanlan (74) prepared epoxy compounds of several of the unsaturated acids and their methyl esters by the use of peracetic acid at temperatures below 25°. Because the ring structures in these compounds open rather rapidly on standing with the reagent, it was necessary to isolate the product immediately after the two to four hour reaction period. They described a new class of oils—epoxidized oils.

Bolland & Koch (75) found the primary product of the action of molecular oxygen and ethyl linoleate to be a monohydroperoxide, which by spectrographic analysis was shown to contain 70 per cent of conjugated diene isomers. One of the first decomposition products is ketonic in nature. Weak absorption was shown at 2750 Å, and intense absorption at 2315 Å which is removed by adsorption on alumina. Mueller & Rusch (76) found that autoxidation of linoleic and linolenic acids is inhibited by benzopyrene in concentrations of 0.2 and 0.3 per cent.

Holman and co-workers have reported four investigations which have employed the spectrophotometer in following the oxidation of pure fatty acids, their esters, and certain fats. In the first of these (77), oxidation of nonconjugated trienoic acids was accompanied by an increased absorption with maxima at 2350 Å and 2750 Å; and oxidation of conjugated trienoic acids was accompanied by decreased absorption at 2600 to 2800 Å and increased absorption at 2300 Å and above 3200 Å. Since the spectra of purified fatty acids of both classes are not affected by cold alkali, they concluded that the absorption bands which appear with autoxidation are due to oxygen-containing chromophores. Later (78), they reported that the ultraviolet absorption at 2750 Å of lard in various stages of autoxidation increases in proportion to rise in peroxide value but is not due to peroxide. Prolonged oxidation of ethyl oleate and elaidate showed marked increases in absorption. These changes were believed to be due to conjugated systems containing carbonyl groups or to conjugated polyenes formed by enolization of these systems. In a further study on highly purified linoleic acid (79), they showed that the autoxidation products of linoleic acid are spectroscopically unlike those of oleic acid. In the latest of their reports (80), oleic acid, ethyl oleate, and certain *cis-trans* isomers of

unsaturated acids were shown not to be substrate for lipoxidase. Linoleic and linolenic acids, their ethyl esters, and methyl arachidonate with lipoxidase showed increased absorption at 2300 Å and 2700 Å, due to conjugated groupings between carbonyl groups and carbon to carbon double bonds.

Oxidative changes in shortenings and other fats were also followed by Filer and co-workers (81) by chemical and spectrophotometric methods. Concurrent with the appearance of appreciable amounts of peroxide, decreases were observed in linoleic acid, total unsaturation, and mean carbon chain length, along with probable formation of conjugated unsaturation. In a study of the stability of soybean flours (82), oxidation of the oil in these flours was shown to be due mainly to loss in diene (linoleic) acid.

CHEMISTRY OF THE SYNTHETIC AND NATURAL GLYCERIDES

Feuge *et al.* (83) esterified mixtures of fatty acids with glycerol to a free fatty acid content of about 3 per cent by heating at 175° for six hours with tin chloride as a catalyst, at 200° with zinc chloride, and at 250° without catalyst. A pressure of 20 mm. is maintained during the reaction to remove water.

Singh (84) described the synthesis of a series of mono-, di-, and triglycerides. Twelve triacid fully saturated glycerides were prepared by Chen & Daubert (85) in a continuation of the work from this laboratory on the properties of glycerides of known structure. Physical properties included in this report are: refractive indices, melting points of polymorphic forms, and solubilities at 25° in ethyl ether, petroleum ether, acetone, and ethyl alcohol. Transition point data were reported (86) for other series of synthetic glycerides, including unsymmetrical mono-oleyl-disaturated glycerides and monosaturated-dioleins and also for a series of saturated 2-monoglycerides. With the last series no evidence was found of polymorphism, although previous work on the 1-monoglycerides had shown this phenomenon. Bailey *et al.* (87) investigated polymorphism and the x-ray diffraction characteristics of tristearin and of hydrogenated cottonseed oil. A new pattern with two short spacings and a long spacing was observed with tristearin. Four forms of the hydrogenated oil were detected. The pattern of the highest melting form was different from either tristearin or 2-palmityl-distearin. Dilatometric data on glycerides were also reported (88, 89).

Relatively few papers have appeared on the glyceride structure of natural fats. One of the most interesting of these, by Hilditch &

Meara (90), is concerned with the glyceride structure of human-milk fat, analyses of the component fatty acids of which were reported from two laboratories last year (37, 91). This investigation included a separation of 430 gm. of the fat into five fractions by crystallization from acetone and study of the glyceride structures of the fats in these fractions by the usual methods. From the results human milk fat was shown to contain 24 per cent of diunsaturated glycerides, in which the saturated acid was capric, lauric, or myristic; 19 per cent of palmityl-diunsaturated glycerides; 20 per cent glycerides containing one palmityl, one unsaturated, and one of either C_{10}, C_{12}, or C_{14} acids; and about 14 per cent of monounsaturated-palmityl-stearins. The proportions of monopalmityl-glycerides and of glycerides containing one radical of either of the lower saturated acids resemble the proportions of the corresponding groups of glycerides in cow-milk fat. Hence, the authors suggest that the origins of the short chain glycerides may be similar in both fats.

In a study of the glycerides of tobacco-seed oil it was shown (92) that the oil contains 7 per cent trilinolein and 35 per cent of oleyl-dilinoleins, in addition to other mixed glycerides, the results being based mainly on preparation and crystallization of the bromine addition products. This oil has been shown by Riemenschneider *et al.* (93) to contain 75 per cent linoleic acid by the thiocyanometric method and somewhat larger amounts, from 3 to 4.5 per cent more, by the spectrophotometric method. Meara (94) by transition-point data and melting points showed that the oleyldistearins from six seed fats consisted almost exclusively of symmetrical 2-oleyl-distearin, and that (95) the palmityl-distearin, prepared by crystallizing hydrogenated cacao butter, cottonseed oil, and pig-back fat, is the 2-isomer. He proved the virtual exclusive occurrence of 2-palmityl-oleyl-stearin (I) and 2-palmityl-diolein (II) in cacao butter, both I and II in pig-back fat and 2-palmityl-oleyl-linolein and 2-palmityl-dilinolein in cottonseed oil.

Jack & Henderson (12) crystallized butter fat successively from pentane at four temperatures: ($-7°$, $-13°$, $-23°$, and $-53°$). Four crystalline fractions and a filtrate fraction were obtained. The fatty acid composition was evaluated for each of the five fractions, the first and last being regarded as fully saturated and fully unsaturated glycerides respectively. From their data, curves were drawn describing the distribution of the saturated and unsaturated fatty acids in each of the five glyceride fractions. They noted the curious result, that the crystalline fraction obtained at $-53°$, melting at $11.4°$, showed a

fatty acid distribution very similar to that of the original fat, which melted at 32.4°.

Fatty acid analyses of the milk fats of the goat, ewe, and mare were made by Hilditch & Jasperson (96). Mare-milk fat was found to differ notably from the others with respect to the high iodine number (164.0) of the unsaturated C_{18} esters. This was due to the presence of about 40 per cent of linolenic acid (14 per cent of original esters). This finding is specific for linolenic acid in that it is based on the yield of ether-insoluble bromides obtained from the free acids. In this fat, as in most other milk fats, no linoleic acid was found, but rather isomeric octadecadienoic acids which yield no tetrabromides melting at 114°.

De la Mare & Shorland (49) in the course of several analyses of lard (back fat) by the ester-fractionation method report considerably larger amounts of C_{20-22} acids than can be accounted for from the arachidonate content as evaluated from the polybromide numbers of the ester mixtures in question. In the course of this work the higher fractions of the unsaturated esters were redistilled and the polyethenoic esters were concentrated by crystallization. Later they reported (97) working up 4 kg. of the fat. They found spectroscopically, after alkali isomerization, eicosadienoic acid to the extent of 0.2 per cent of original fat. Not over 5 per cent of the C_{20} esters was di- and polyethenoic, so that they concluded that the fraction was mainly eicosenoic acid. They further reported (98) the presence of 13 molar per cent of myristic acid in the back fat of pigs fed on diets supplemented with copra. Fast growing pigs assimilated dietary linoleic acid to a greater extent than slow-growing pigs, while myristic and lauric acids appeared more quickly in the depots of fast-growing animals. The C_{18} acids of the lard of milk-fed pigs contained no linoleic acid. The differences in unsaturation in outer back fat, inner back fat, and perinephric fat of pigs were found to be due mainly to differences in the ratios of stearic to oleic acid, differences in linoleic acid content being negligible (99).

Analyses of the fatty acids of two important cereal oils were reported since last year's review. Wheat-germ oil was shown by Thaler & Groseff (100) to contain 57.4 to 61.2 per cent linoleic acid and 1.6 to 4.3 per cent linolenic acid. An analysis of this oil by Radlove (101) gave the following acid composition: saturated acids 15.5; oleic 25.5; linoleic 52.6; and linolenic acid 6.3 per cent. The value for linolenic acid was obtained by thiocyanometry and was checked by

determining the yield of ether-insoluble hexabromides, from which a slightly higher value was obtained, 7.2 per cent. Corn oils from Bulgaria and Argentina (102) showed quite different compositions. Linoleic acid contents were 60.7 and 40.8 per cent, respectively and linolenic acid contents, 2.7 and 1.5 per cent. Baur & Brown (103) found 56.3 per cent linoleic acid in a specimen of American corn oil; this oil contained only traces of linolenic acid. An unusual drying oil, hip-seed oil, has been described by Steger & van Loon (104). It contains 54.2 per cent linoleic acid, 32.0 per cent linolenic acid, and only 5.4 per cent saturated acids. Similar to this in composition is stillingia oil (105) which contains 66.8 per cent linoleic, and 21.4 per cent linolenic acids.

Holman *et al.* (106) in an attempt to identify the coloring matter in oxidized rancid fats when treated with alcoholic alkali found that the compounds causing this phenomenon originate from unsaturated fatty acids and are related to colored compounds derived from chroman-5,6-quinone by reaction with alkali.

The Phospholipids

Gortner (107) obtained comparable phospholipid values in a series of lipid extracts of pig embryo tissues by three methods: determination of lipid phosphorus and multiplying by the factor 24, Bloor's oxidation method for phospholipid fatty acids, and direct oxidation of phospholipid. Artom (108) has devised a method for quantitatively differentiating ethanolamine and serine, and has applied the procedure to the determination of phosphatidyl ethanolamine and serine in rat tissues and human plasma. Serine-containing phospholipids were found in all materials analyzed, but they represent only a minor fraction of the total and noncholine-containing phospholipids. The following values in μM per 100 cc. were obtained for human plasma: total phospholipids, 197; choline-containing phospholipids, 131; ethanolamine-containing phospholipids, 42; and serine-containing phospholipids, 13. Taurog *et al.* (109) had previously reported the following values in mg. per 100 cc. for human plasma: total phospholipids, 235; choline-containing phospholipids, 229; and noncholine-containing phospholipids, 6. Welch (110) described a modified procedure for estimating α- and β- lecithins and cephalins in tissues. The α forms predominate in the brain and α-lecithin and β-cephalin in the heart and liver.

An analysis of salmon roe (111) gave 12.5 per cent glyceride and

6.2 per cent phospholipid. Although the body fat of rats fed castor oil contained 7 per cent ricinoleic acid, Stewart & Sinclair (112) found the phospholipid of the liver, small intestine, and muscle not to contain this acid. The observation that the brain contains acetylcholine bound in a phosphatide complex was further verified (113) by the demonstration that cobra venom, heated at 100° for twenty minutes (which inactivates proteinase but not lecithinase), liberates lysolecithin, acetylcholine, and a fatty acid from a brain-tissue suspension. Fairbairn (114) studied the phospholipase of the cottonmouth moccasin. The enzyme hydrolyzes both lecithin and cephalin, but has no effect on cerebrosides, sphingomyelins, acetal phospholipid, and lyso-phospholipid. The normal free fatty acid content of tissues was found by Fairbairn (115) to be low, but autolysis even for a short time increases the amount present. Within a few minutes after extirpation of rat or cat livers, one twelfth of the constituent phospholipid is hydrolyzed by the intracellular phospholipases. Fatty livers of rats, fed high-fat, choline-free diets (116), showed an increase in cephalin, but the choline-containing phospholipids were the same as in rats receiving 5 mg. choline per day. Adrenalectomy caused loss of fat without affecting the phospholipids of the liver. The choline-phospholipid/cephalin ratio was unchanged during fasting during which neutral fat disappeared and phospholipid decreased. The increase in cephalin may result from insufficient methylation with consequent slower lecithin synthesis. Pangborn (117) described a simplified procedure for the preparation of cardiolipin which is used in serodiagnostic tests for syphilis; the purification of lecithin is also discussed. Further references to phospholipids in association with other lipids are found in the section which follows.

THE TISSUE LIPIDS, NORMAL AND PATHOLOGICAL

The necessity of breaking down lipid complexes (lipoproteins), preliminary to complete extraction of lipids with such solvents as ether, chloroform, and the like, has been emphasized again. Grossfeld & Hess (118) found that yeast lipids are not extracted with solvents without preliminary treatment with hydrochloric acid; Reichert (119) observed that less than half of the lipids of yeast are extracted by solvents. Refluxing with 95 per cent methanol breaks up the complex and allows subsequent complete extraction. Direct extraction with acetone is recommended by Nielson & Bohart (120), total lipid being regarded as the petroleum ether-soluble portion of the acetone extract.

Experiences with extracting the lipids of dried eggs are described by Brooks & Hawthorne (121).

Hunter *et al.* (122) reinvestigated methods for determination of tissue lipids. This work includes a critical examination of methods of extraction and saponification, a titrimetric method for determination of total fatty acids, and a modification of the Schoenheimer & Sperry method for free and ester cholesterol along with a study of the best conditions for precipitation of digitonide and development of color in the Liebermann-Burchard reaction.

Kountz *et al.* (123) observed that although men become atherosclerotic earlier and more frequently than women, the blood cholesterol levels of women are 237 and of men 196 mg. per 100 cc. The acid precipitable cenapses of blood serum (124) are complex compounds of phospholipid, sterol, and protein; electrophoretically separated fractions were shown to be of the same composition. Tayeau (125) suggested that the lipids and protein in these compounds are held together by forces of the Van der Waals type. Fractional blood lipid determinations (126) in dogs fed diets in which the fat was egg-yolk lipid·or lard showed several effects. On high egg-yolk diets there was a temporary elevation of cholesterol, choline phospholipid, and total phospholipids. Lard caused a moderate sustained depression of lipid fractions. A comparison (127) of the lipids in maternal and fetal blood plasmas of sheep showed parallel values; the fetal lipid values were one-half to two-thirds the maternal. Detailed analyses of plasma lipids of normal persons and persons with tuberculosis (128) showed the following fractions to be significantly higher in normal persons: total phospholipids (165 vs. 116 mg. per 100 cc.); lecithin (62 vs. 31); cephalin (96 vs. 80); total cholesterol (132 vs. 106); free cholesterol (42 vs. 28); phospholipid fatty acids (111 vs. 78). Tuberculous plasma lipids were higher in total fatty acids (361 vs. 298). Studies have also been made on the serum (plasma) lipids in rheumatoid arthritis during gold salt therapy (129), in celiac syndrome (130), and in starvation and water deprivation (131).

Three studies of the immunological properties of specific glucolipids have appeared (132, 133, 134). An important contribution to the chemistry of the lipids of the nuclei of liver cells has been made by Williams and co-workers (135). The functional role of these lipids is structural rather than metabolic. The lipid pattern of the nucleus is similar qualitatively to that of whole liver tissue but differs quantitatively from it. The structural lipids comprise 12 to 14 per cent of

the dry weight of the nucleus or three-fourths of the total lipid. Ninety per cent of the structural lipid is phospholipid, of which less than 5 per cent is sphingomyelin. Nuclei isolated from cancerous liver cells (produced by feeding butter yellow) have a greatly reduced phospholipid content and increased ester cholesterol. Derangement of phospholipid content is observed essentially in the nucleus. Claude (136) found that the mitochondria from leukemic cells of rats, isolated by centrifugation at relatively low speed, contained about 27 per cent lipids, of which about 75 per cent is phospholipid. Microsomes contained 36.6 per cent lipids.

An interesting new tissue lipid, dicholesteryl ether, has been isolated (137) for the first time from a natural source, beef spinal cord, in which it occurs to the amount of 1.5 to 2.0 per cent. The carotid ganglion of the horse was found (138) to contain 7.42 per cent lipids (lecithins 7, sphingomyelins 14, and galactolipids 78 per cent). A unique contribution to nervous tissue chemistry is that of Patterson *et al.* (139) who found the lipids of the central nervous system of the honey bee to be of the same types and in about the same proportions as those in the brains of vertebrates, with the exception of the cerebrosides and the low sterol content in the bee.

Jones & Peck (140) found that 42 per cent of a series of tuberculous patients coming to autopsy showed fatty infiltration of the liver. Zwemer & Wotton (141) fed guinea pigs test meals of cod-liver oil stained with Sudan IV and sacrificed the animals at constant intervals. Histological examination of sections of frozen kidneys gave evidence for excretion and resorption of fat. The problem of fat phanerosis, or "unmasking" of fat, has been attacked by Popjak (142), using human post-mortem kidneys as experimental material. Both histological and chemical data are included. The cortex of adults gave a mean value for total fatty acids plus cholesterol (by saponification) of 1.72 per cent; the medulla, 1.26 per cent. Fatty change occurred more often in the medulla than in the cortex. Chemical analysis showed this change to be brought about by infiltration of neutral fat from the depots. Phospholipid and free and ester cholesterol showed no changes. Cortex had a higher phospholipid content than medulla; also, kidneys of infants had more phospholipids than those of adults. Fatty degeneration in this organ appears to be fat infiltration.

Long & Fry (143) found that subcutaneous or intravenous injection of epinephrine caused a definite decrease in adrenal cholesterol in rats, an effect not produced in hypophysectomized animals. On the

other hand, Carreyett, Golla & Reiss (144) observed that, while un-treated male Wistar rat adrenals contained 2 to 3 per cent cholesterol, this value was raised 80 per cent by administration of the gonadotropic hormone and was reduced by the corticotropic hormone of the pituitary. A marked reduction of stainable lipid was observed (145) in rat adrenal lipids after crushing blow shock, due to loss of ester cholesterol.

Gortner (146), in a study of the lipids of the pig during embryonic development, found that total lipid and the lipid-protein ratio remained constant for most of this period. A large part of the "neutral fat" fraction of lipids may be present as free fatty acids. The very young fetus has twice as much phospholipid (dry weight basis) as does the fetus at term. Unsaponifiable lipids progressively decreased. Glycerides appear at about the middle of the gestation period, but even at term they account for only a small part of the total lipid.

Mitchell *et al.* (147) analyzed a human cadaver. Ether extract values (per cent) for the several tissues were: spleen, 1.19; lungs, 1.54; muscle, 3.35; kidneys, 4.01; alimentary tract, 6.24; liver, 10.35; brain, cord, and nerve trunks, 12.68; skin, 13.0; pancreas, 13.08; skeleton, 17.18; adipose tissue, 42.44. The lipids made up 12.51 per cent of the whole cadaver. This value is interesting in comparison with the results of estimation of total fat content of the human body from the equation of Rathbun & Pace (148). Body fat, per cent $= 100 \ [(5.548/sp.g.) - 5.044]$. Specific gravities of 1.002 to 1.100, substituted into this equation give fat contents of 49.3 to 0 per cent.

Wicks & Suntzeff (149) found that after a single application of carcinogenic methylcholanthrene in mice the ratio of cholesterol to protein nitrogen (normal 0.27) of the skin falls for about ten days and corresponds closely to the changes previously noted in the total lipid/protein nitrogen ratio. Wynn & Haldi (150) observed that in rats on a high-fat diet the fat content of the skin almost doubled in one to three weeks along with decrease in water content. The acetone-soluble fat obtained by Velick (151) from transplanted rat tumors, initiated by *Cysticercus fasciolaris,* is composed of ordinary glycerides, free fatty acids, and 28 per cent of unsaponifiable material which is mostly cholesterol. This fat was high in linoleic and in C_{20-22} unsaturated fatty acids. The lipids from enlarged prostate glands were studied by Scott (152); total lipid was low, as was the phospholipid/cholesterol ratio; there was no neutral fat. The main phospholipid was cephalin.

An anticoagulant lipoprotein has been isolated from various tissues by DeSuto-Nagy (153); it is found in the albumin fraction, the protein being an inert carrier of the lipid which behaves like a sphingomyelin. Chargaff (154) prepared thromboplastic protein of very high particle weight from human placenta and lungs. The lipoprotein from placenta contained 39.7 per cent lipid and from lungs, 53.3 per cent. The iodine numbers of the purified lipids from these sources were 74.0 and 63.8 respectively. Hanson *et al.* (155) found that the only antioxidants deposited in the depot fats are compounds having the properties of vitamin E. Okey (156) has reported analyses of a large number of animal foods for total fatty acid and cholesterol; also analyses are given of tissues of a number of laboratory animals, including the rat (on three levels of dietary fat). She notes that the only animal tissues, commonly employed as foods, with cholesterol contents higher than 1 per cent are brain and egg yolk.

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THE CHEMISTRY OF THE PROTEINS AND AMINO ACIDS

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Following the development of precise physical methods for the characterization of proteins, new ways for their separation and purification were devised (1, 2). Several well characterized proteins are now available for studies of composition and behavior. During the period covered by this review notable advances have been made in the amino acid analysis of proteins. The development of biological methods of assay as well as of other new methods has provided new techniques which are of particular importance in determining the neutral amino acids—a group which presented an all but insoluble quantitative problem when studied by the older methods of analysis. Complete amino acid analysis of proteins is now being attained, more than 95 per cent of the total residues of several proteins having been accounted for (3, 4). The value of this data for the interpretation of physical and chemical properties of proteins and as a basis for structural considerations cannot be overestimated. This work has been discussed in a series of reviews presented at a conference at the New York Academy of Sciences (4a).

Protein Purification and Analysis

Purification of proteins.—Studies on the purification and interaction of the muscle proteins have been carried out with remarkable success by Szent-Györgyi and his associates (5, 5a). Myosin, the principal protein of muscle, was isolated in the crystalline form as a potassium complex from solutions containing a low concentration of potassium chloride. It is believed that myosin as prepared by previous investigators was combined with varying amounts of a newly discovered protein, actin, which forms the insoluble portion of muscle. The concentration of myosin and actin in muscle is 8 and 3 per cent respectively. Crystalline myosin was found to be soluble in water and has a strong double refraction of flow which is due to association of myosin particles since it is abolished by salt. Crystalline myosin showed the same adenosinetriphosphatase activity as the impure preparations. The

specific viscosity of actin solutions is low and they do not show double refraction of flow. However, if a dilute solution of potassium chloride is added to a neutral solution of actin, the solution develops turbidity, becomes highly viscous, and shows double refraction of flow. Thus potassium chloride converts the globular actin into a fibrous form. The fibrous form may be reverted to the globular form by removing the salt by dialysis. It is thought that globular actin polymerizes to give the fibrous form. The globular form is stable at pH 7.0 to 7.5, but is destroyed even at $0°$ when the solution is more acid or alkaline. When dilute solutions of myosin and fibrous actin are mixed a viscous substance, fibrous actomyosin, is formed, the properties of which vary with the proportions of the components. The most striking property of fibrous actomyosin is its contraction and dehydration in the presence of salt and adenosinetriphosphate. It can be dissociated into globular actin and myosin. A molecular theory of muscular contraction based on the properties of myosin and actin and their interactions with adenosinetriphosphate and inorganic ions has been developed. The relationship of myosin to adenosinetriphosphatase has been investigated (6). The results are in apparent disagreement with the view that these substances are identical.

Lysozyme has been isolated from egg white and obtained in the crystalline form (7). Considerable purification was accomplished by adsorption on bentonite. Crystallization could be accomplished over a pH range from 4.0 to 11.0 by means of sodium chloride or ammonium sulfate, advantage being taken of a large temperature coefficient. It appears to be homogeneous by both electrophoresis and the ultracentrifuge. The isoelectric point was found to be at pH 10.5 to 11.00 and the molecular weight to be about 17,000 by osmotic pressure and the ultracentrifuge. The crystals of lysozyme hydrochloride are large and the optical properties and water content have been determined (8).

Rennin, the milk clotting enzyme, has been crystallized and purified until it gives a constant solubility (9). Crystalline rennin has proteolytic activity with a pH optimum of 3.7; however, impure preparations contain pepsin with a pH optimum of 2.0. It is implied that the difference in pH optimum for proteolysis is sufficient to distinguish rennin from pepsin. Purified casein, a much studied protein, was shown to be contaminated with a proteolytic enzyme (10). The separation, functions, and clinical uses of the blood proteins have been summarized (11). A low density protein was isolated from human serum by means of ammonium sulfate fractionation. After re-fractionation,

the substance was homogeneous by electrophoresis (12). The seroglycoid fraction of serum described by Hewitt was shown to consist of three or four proteins by fractional precipitation with phosphate (13). The two least soluble components were recovered in the pure form.

Parallel studies were made on crystalline hemoglobins and myoglobins of horse, dog, and man (14). Myoglobin as judged spectrophotometrically by conversion into hemochromogen is unusually stable towards alkali as compared to hemoglobin. Crystalline synthetic (reconstituted) myoglobin was prepared from heme and globin; it showed all of the spectrophotometric characteristics of ferrimyoglobin (15). The molecular dimensions of hemoglobin were compared with the dimensions of other cellular components (16).

The d-glyceraldehyde-3-phosphate dehydrogenase of rabbit muscle was isolated and crystallized from ammonium sulfate (17). A yield of 7 per cent of the extracted proteins of muscle was obtained which was crystallized and was found to be electrophoretically homogeneous (18). The PR (prosthetic group removing) enzyme which catalyzes the enzymic conversion of phosphorylase-a to b was purified (19). Manganese ions are necessary for the conversion. A prosthetic group containing organic phosphorus is split off in the reaction. Phosphorylase-a (previously crystallized) was converted into phosphorylase-b by the PR enzyme and the phosphorylase-b was then crystallized by means of ammonium sulfate (20). The alkaline phosphatase of kidney has been isolated and crystallized by means of acetone water mixtures at $0°$ (21). Hemolysin (lecithinase) from cobra venom was crystallized (22, 22a). It has an isoelectric point of 8.5 to 8.6 by electrophoresis and a molecular weight of 31,600 as determined by diffusion.

The proteins of plants have been recently reviewed (23). A trypsin inhibitor, present in soybeans, was crystallized (24). Adsorption on bentonite and elution with pyridine was used effectively as in the purification of lysozyme (7). Trypsin inhibitor from soybean is a larger molecule than the inhibitor present in the pancreas gland. The crystallization of southern bean mosaic virus was reported (25). Purification was accomplished by centrifugation and further separation effected by means of salts and alcohol. It was found to be homogeneous with respect to electrophoresis and the ultracentrifuge.

The separation on a large scale of the leaf proteins of several plants was reported, as well as analysis of the separated proteins for some of

the amino acids (26). A protein, linin, which is the major component was isolated from flaxseed and appears to be homogeneous by the solubility method (27). Ethylene glycol and dioxane in high concentrations, as well as strong formic acid, were used in the purification. Gluten was fractionated from acetic acid-ethyl alcohol solution at different temperatures into three arbitrary fractions (28).

The proteins of peanut meal have been investigated electrophoretically (29, 30). Neither arachin nor conarachin (31) was found to be homogeneous by this method although arachin contains none of the slowly migrating minor components of the meal. These minor constituents are appreciably soluble at the isoelectric point and were prepared free of the major components which are present in both arachin and conarachin.

A new globulin, fetuin, has been isolated from calf serum by means of fractional precipitation with ammonium sulfate (32). It was precipitated when the ammonium sulfate concentration reached 37 to 40 per cent saturation. In a new-born calf this globulin amounts to 20 per cent of the total serum protein and in a six month old calf to 5 per cent of the total serum protein. The extrapolated value of the sedimentation constant was $S_{20} = 3.29$. The diffusion constant was $D_{20} = 5.5 \times 10^{-7}$, giving a molecular weight of 51,000. Its behavior is equivalent to that of a prolate ellipsoid with an axial ratio of 11.

Prothrombin has been prepared free from antithrombin by means of ammonium sulfate precipitation (33). From solubility measurements it was deduced that the preparation was homogeneous. The substance contained 4.3 per cent carbohydrate and 10 per cent tyrosine.

Protein antigen of tubercle bacillus has been extracted from the water-insoluble residue of the dried bacillus with dilute sodium hydroxide (34). The extracts had a high tuberculin potency and the presence of two or three components was indicated by ultracentrifugal and electrophoretic experiments. The proteins of the tubercle bacillus have also been the subject of a recent review (35).

A hemoglobin-like protein has been isolated from the nodules of soy beans (36). The substance acts as a reversible oxygen carrier and not as a catalyst of the cytochrome type. A hemoglobin with a very high affinity for oxygen has been found in the body walls and one with still higher affinity in the perienteric fluid of *Ascaris lumbricoides var. suis* (37).

The anterior hypophyseal growth hormone has been isolated from

the pituitary glands by means of salt fractionation and isoelectric precipitation (38). The homogeneity of the substance was demonstrated by means of electrophoresis and solubility determinations. The isoelectric point is at pH 6.85 as determined by electrophoresis. The molecular weight in the presence of ammonium sulfate was found to be 44,250. The growth hormone is not inactivated by urea in high concentrations.

Amino acid analysis.—A considerable increase in interest in the amino acid analysis of proteins is evident. This is in part due to the development of biological methods of assay which are rapid and specific and require only a small amount of material for analysis. Most of these methods are based on the growth response of an organism of the *Lactobacillus* group to added increments of an amino acid when this acid is not supplied in the basal medium. The response is measured either by titrating the lactic acid produced or by measuring the turbidity resulting from growth of the organism and is compared with a standard curve relating the response to known concentrations of the amino acid. The subject has been thoroughly reviewed by Snell (39, 39a). Analyses on protein hydrolysates have been reported recently by this method for aspartic acid (3, 40, 41), glutamic acid (3, 42 to 45), arginine (46, 47), histidine (47, 48), lysine (3, 47, 49), glycine (3), alanine (3), valine (3, 46, 47, 50), leucine (3, 47, 50, 51), isoleucine (3, 47), phenylalanine (3, 47), tryptophane (47, 52, 53), methionine (47), proline (3), threonine (47), and serine (3, 41). In addition it has been shown that for some of the organisms tyrosine and cystine are essential for growth (54) although no detailed methods or bioassays have been reported for these acids. Thus, of the commonly occurring amino acids only hydroxyproline and cysteine have not been found essential for the growth of some microorganisms. In general the values obtained by the microbiological method are in good agreement with the results obtained by chemical methods.

Biological assay methods have also been developed for leucine (55) and lysine (56) utilizing the growth response of mutant strains of the mold *Neurospora*. In this case different mutants are employed for the analysis of each amino acid and the acid in question is the only one required as an amino acid supplement for growth of the particular mutant used. Growth is measured by determining the dry weight of the mycelium formed after incubation under standard conditions.

Another type of biological assay for amino acids is that of Gale in

which a microorganism is used as a source of a specific amino acid decarboxylase. The carbon dioxide produced by the reaction is measured manometrically and a small correction is applied for incomplete decarboxylation as determined on known amounts of the amino acid. Some analyses are reported for lysine, histidine, tyrosine, glutamic acid, and ornithine (57 to 60). A specific decarboxylase for arginine has also been reported (61).

Some new analyses by the isotope dilution method have been reported for crystalline bovine and human serum albumin (62) and for horse carboxyhemoglobin and β-lactoglobulin (63). This method in common with the biological determinations is specific for the naturally occurring isomers of the amino acids. Thus if racemization takes place during hydrolysis the values obtained will be low. However Foster (63) points out that the isotope dilution method can be used to determine the extent of racemization in the hydrolysate. He reports an experiment on the determination of phenylalanine in which samples of both the *l*- and *dl*-forms were isolated after the addition to the hydrolysate of *dl*-phenylalanine containing excess N^{15}. From the values for atoms per cent excess N^{15} on these samples he calculated that phenylalanine was racemized 1.8 per cent during hydrolysis. Racemization of glutamic acid has been shown to be less than 1 per cent (64). It is not known whether other amino acids are racemized to the same extent or whether there is considerable individual variation.

The destruction during hydrolysis of an amino acid in the free state can be determined by the isotope dilution method, but if this differs from the destruction when the amino acid is combined as a peptide the total extent of destruction during protein hydrolysis may not be determined (65). This problem becomes of considerable importance in estimating serine [see for example (3, 4)] and presumably could be solved only if it were possible to determine some specific product of the decomposition in the hydrolysate.

Colorimetric methods have been devised or existing methods refined for arginine (66), tryptophane (67), tyrosine (68), and histidine (69). Amino acid analyses are reported for salmine (70) and human hair keratin (71).

The determination of amino acids by partition chromatography has been reviewed by Martin & Synge (72) and Cannan (73) and a new indicator described for use in this method (74). A chromatographic method for the separation of glutamic and aspartic acids has

been described employing aluminum oxide as the adsorbent (75). Another method for separating amino acids makes use of their adsorption on silver sulfide (76). Further work on adsorption of amino acids and peptides on active carbon (77) and an automatic recording device for this type of adsorption analysis (78) are reported. Chromatographic adsorption of amino acids on ion exchange resins has been studied (79). The adsorption in this case is dependent on the acid-base properties of the acids and not on the nature of the side chains, as in the type of chromatography employing distribution between immiscible solvents or on a nonexchanging adsorbent. It was concluded that more work is necessary before these resins can be used for quantitative separation into acidic, basic, and neutral amino acids although the separation of the dicarboxylic acid fraction from protein hydrolysates has been accomplished (80).

A new method has been described for the identification and estimation of the free amino groups of insulin and other proteins (81). A derivative of insulin and 2,4-dinitrofluorobenzene was prepared which gave only 0.04 per cent free amino nitrogen by the Van Slyke method. After hydrolysis with acid, the 2,4-dinitrophenylamino acid derivatives were separated by chromatographic partition and estimated colorimetrically. The 2,4-dinitrophenyl derivatives of pure amino acids were prepared and their rates of destruction determined when treated under the conditions of protein hydrolysis. Corrections due to destruction during hydrolysis were applied to the results on insulin. It was found that an equivalent weight of 12,000 contained two glycine and two phenylalanine residues containing free α-amino groups and two lysine residues containing free ε-amino groups. It had been previously shown by a related method that the amino groups of phenylalanine in insulin are free (82). It is suggested that a sub-molecule of minimum molecular weight of 12,000 is composed of four open peptide chains held together presumably by disulfide links.

The methods for determination of amino acids and the amino acid composition of several proteins have been recently reviewed (4). Purified amino acids in known mixtures simulating the composition of insulin were determined with a high degree of accuracy with the exception of glycine and hydroxyproline which were not determined. The determined nitrogen accounted for 96.7 per cent of the total nitrogen in casein, 92.2 per cent in gliadin, 94.0 per cent in egg albumin, 93.1 per cent in gelatin, 83.3 per cent in collagen, 96.4 per cent in insulin, and 97.8 per cent in edestin. The previous estimate

of eighteen terminal amino groups per molecule of insulin of a molecular weight of 36,000 is revised in favor of the more recent direct determination (81) of twelve terminal amino groups per molecule. It is assumed that the higher values obtained by subtraction of the ε-amino nitrogen of lysine (Van Slyke method) from the total amino nitrogen on the intact insulin molecule are erroneous since glycine peptides give abnormally high values for amino nitrogen by the Van Slyke procedure. The hypothesis is advanced that there are four chains in each submolecule of minimum molecular weight 12,000. Presumably there is an equal number of terminal carboxyl groups. Evidence for the presence of terminal carboxyl groups is consistent with the titration curve of insulin (83). The new results on edestin indicate that it is composed of one chain per equivalent weight of 50,000 since it has only one end group for each equivalent. Hydroxylysine was found in gelatin and collagen but not in casein, zein, gliadin, and egg albumin.

The composition of β-lactoglobulin has been reported to a remarkable degree of completeness (3). The theoretical total amount of amino acids of this protein are accounted for within 1 per cent. The amino acid data are most consistent with a molecular weight of 42,020. Tryptophane and cysteine analyses are determinants in obtaining this value. These results are in agreement with results obtained by other methods recently reported (4), with the exception of serine and threonine. Both of these amino acids decompose during hydrolysis and a correction is applied. It is concluded that β-lactoglobulin consists of 370 amino acid residues with 366 peptide bonds arranged in four subunits or polypeptide chains.

Some of Thudicum's original preparations of "glycoleucine" have been examined by partition chromatography and have been shown to consist of dl-leucine (84). This result was substantiated by selective oxidation by an l-amino acid oxidase and by asymmetric resolution employing a microorganism, d-leucine being identified following both of these procedures. The properties of the acetyl derivatives were in agreement with those of dl-leucine. It was the study of a similar amino acid fraction that led to the claim that norleucine is a constituent of nervous tissue proteins (85). An acid hydrolysate of ox spinal cord was examined chromatographically. The absence of norleucine was indicated although when authentic norleucine was added to the hydrolysate the presence of the added substance could easily be demonstrated. It was concluded that norleucine could not be present in an amount equivalent to more than 0.03 per cent of the total nitrogen.

The occurrence of norleucine as a constituent amino acid of proteins is thus open to question.

Analytical procedures on proteins.—A method has been devised for determining protein concentration by means of the biuret reaction (86). Ethylene glycol is used to prevent the precipitation of copper hydroxide and gives clear solutions which can be used for the colorimetric determination of protein. The separation of the albumin and globulin fractions of human serum by methanol appears to be more satisfactory than the frequently used sodium sulfate method (87). The correlation between the albumin-globulin ratio, obtained by means of the alcohol separation, with the electrophoretic determination is good, while sodium sulfate separation gives a poor correlation.

An examination has been made of the conditions necessary to obtain accurate nitrogen values on amino acids and several proteins (88). Both the digestion and distillation procedures were studied. The much used digestion mixture consisting of potassium sulfate, mercuric oxide, and sulfuric acid was found to be satisfactory if the protein was digested for a period of five hours, followed by the addition of a drop of alcohol and digestion for an additional hour.

PROTEIN PROPERTIES AND STRUCTURE

In continuation of previous work on the elasticity of keratin fibers (89) a study has been made of the influence of temperature on the stress at constant stretch over a range of values during both extension and contraction of the fiber (90). The curve for total stress plotted against percentage of extension was similar (at a temperature of 25°) to the usual type of stress-strain curve obtained at a constant rate of loading. The total stress was separated into an element due to internal energy and an entropy term. The latter was calculated from the temperature dependence of the stress and was subtracted from the total stress to obtain the stress due to internal energy. The force of contraction due to the internal energy proved to be the dominating factor in contrast with the elastic element of rubber for which the force due to entropy change is several times greater than the internal energy term.

Monolayers of egg albumin have been studied on 35 per cent ammonium sulfate solution (91). On this medium the protein spreads very rapidly and remains entirely in the surface layer. A molecular weight of 44,400 was derived by extrapolating the values for force

times area to zero film pressure. A similar study of β-lactoglobulin (92) gave a molecular weight of 44,000. Mixed monolayers of egg albumin and lauryl sulfate (93) gave results which were interpreted as indicating the formation of two well defined complexes containing seventeen and thirty-two molecules of lauryl sulfate per molecule of egg albumin.

The dielectric properties of β-lactoglobulin have been studied in glycine solution and in the liquid crystalline state (94). The influence of both glycine and lactoglobulin concentration on the dispersion was sufficiently great to conclude that little significance could be attached to any interpretation of the shape of the molecule based on these measurements. Serum proteins extracted in the dry state with alcohol and ether exhibited considerably altered dielectric behavior (95).

The technique of shadowing objects for examination by the electron microscope by oblique deposition of a film of gold about 8 Å thick (96) has been applied to the examination of some viruses and proteins. Tobacco mosaic virus appears as rods of about 125 Å diameter and of a length which varies continuously from 200 to 1,000 Å (97). Preparations of other plant viruses were deposited in the evaporated film as an ordered array of the elementary particles, essentially a two dimensional crystal, with evidence in some cases of the formation of additional layers to give arrangements more than one particle thick (98). There was some correlation between the shape and arrangement of the elementary particles and the macroscopic form of the crystals of these viruses. In contrast, hemocyanin which is not crystallizable was deposited chiefly as isolated particles or unordered clusters (99).

A theory describing the combination of fibrous proteins with simple acids (100) has been extended to include dye acids, the anions of which have relatively high intrinsic affinities for the protein (101). An alternative treatment of the combination of anions with proteins is that of Steinhardt (102).

Evidence has been presented that the hemocyanin of *Helix pomatia* contains two components (103). One of these is readily dissociated into half molecules by 1 *M* sodium chloride at pH 5.2 and can be obtained in this form as the supernatant liquid when a solution in 1 *M* sodium chloride is ultracentrifuged. The precipitate consists of a mixture of molecules of the original size, presumably the second component, and half molecules. The half molecules can be reassociated by dialysis against dilute salt solution. Both fractions can be dissociated

at high pH into one eighth molecules as had been previously demonstrated (104). On reassociating hemocyanin, after exposure to pH 8.3, by adjusting the pH to 5.3 during dialysis, mixtures of the fragments of the two components appear to be formed. This was shown by the different behavior of the original and reassociated material on exposure to 1 M sodium chloride.

The conversion of globular proteins by heating and stretching to a form giving the β-keratin x-ray diffraction pattern characteristic of fibrous proteins has been studied (105). The diffuse reflections corresponding to spacings of 10 Å and 4.6 Å sharpen on heating in the presence of water. Subsequent stretching of the heated protein in steam or under other conditions in the presence of water results in a fiber showing a high degree of orientation. The atypical "egg white" pattern (106) in which the 4.6 Å reflection is arced on the meridian instead of on the equator was produced in several proteins by giving a low degree of stretch after a relatively short period of heating. X-ray examination of a single large crystal of tobacco necrosis virus derivative was reported (107). The molecular weight calculated from the size of the unit cell and using assumed values for the density and water content of the crystal was found to be about 1,600,000.

The results of an extended ultracentrifugal study on serum and serum proteins has been published (108). A component of the sedimentation pattern of human serum, the "X component," is reported which shows a very marked decrease in sedimentation constant with increase in density of the solvent. The data over a range of salt concentration show this relation to be linear and to extrapolate to $S_{20} = 0$ at a solvent density of about 1.03. The X component and the influence of density on the sedimentation pattern disappeared after extraction of the serum with alcohol and ether. It was concluded that this as well as the high partial specific volume (0.969 for the presumably hydrated particle) was consistent with the presence of combined lipid material. Numerous fractionation experiments with ammonium sulfate were carried out. No fraction was obtained however which was homogeneous in both the ultracentrifuge and the electrophoresis apparatus. Fetuin (32) was prepared in a fairly pure state, but not free from other globulin fractions. Molecular weights reported include fetuin, 50,600; human serum albumin, 72,800; fetal bovine albumin, 67,800; human γ-globulin, 170,000; bovine γ-globulin, 182,000.

The molecular dimensions of zein have been investigated by de-

termination of its double refraction of flow (109). This was made possible for such a relatively compact molecule as zein by the development of a concentric cylinder apparatus capable of producing very high velocity gradients (110) and by the use of solvents of comparatively high viscosity. The results are consistent with a prolate ellipsoidal model about 300 to 400 Å in length and on this basis are in good agreement with sedimentation, diffusion, and viscosity measurements.

A theory of the structure of globular proteins has been presented (111) which pictures the main forces stabilizing the molecules as being due to van der Waals' attraction between the purely hydrocarbon side chains. The magnitude of the van der Waals forces assumed to be operative was calculated from the heat of vaporization of a quantity of hydrocarbon equivalent to the hydrocarbon side chains. It seems improbable that more than a small fraction of this could contribute to the stabilization of the molecule by any possible method of packing the side chains. It has been suggested that γ-glutamyl peptide bonds occur generally in proteins (112). This conclusion was based on the finding that succinic acid, which is produced in small amounts when proteins are oxidized with hydrogen peroxide, was present in larger quantity when the oxidized protein was subsequently hydrolyzed with hydrochloric acid. The additional succinic acid was assumed to come from γ-glutamyl peptides which would be present in the oxidized but unhydrolyzed protein as succinyl peptides.

The solubility of a series of respiratory proteins was determined and the constants in the solubility equation $\log s = \beta' - k'\mu$ were evaluated (113). The constant k' was found to be characteristic of the type of respiratory protein. Artificial nucleoproteins were made by precipitating a protein-nucleic acid mixture with acetic acid (114). The protein was capable of binding any amount of nucleic acid up to 25 to 27 per cent. Experiments with deaminized and acetylated proteins indicated that the capacity of the protein to bind nucleic acid was dependent on free amino groups. Two fractions of gelatin prepared by alcohol precipitation were studied in the ultracentrifuge (115). One fraction with a molecular weight $M_s = 149,300$ was quite polydisperse, while the other fraction (14 per cent of the gelatin) with $M_s = 18,400$ was more homogeneous. A dilatometric method for determining the partial specific volume of proteins in alcoholic solution has been devised (116). Values of 0.776 for zein and 0.724 for gliadin were reported. A study of the distribution of molecular weights of proteins (117) provides no evidence for Svedberg's hy-

pothesis of the occurrence of groupings in molecular weight around certain favored values.

An automatically recording surface balance of the Wilhelmy-Dervichian type for work in the temperature range 0° to 55° has been designed (118).

DENATURATION

Numerous comparative studies of denaturation have been made in which only one property of the denatured protein is considered, such as sulfhydryl groups, with little attention being given to the state of aggregation or degradation of the molecule. In contrast to this approach, a comprehensive study was reported (119) on the preparation and purification of denatured egg albumin. The classical definition of denaturation was adopted, namely, insolubility at the isoelectric point, the native protein being soluble. Solutions of crystalline egg albumin were denatured by acid, alkali, and heat. Purification was accomplished by solution in alkali at low temperatures and low salt concentrations. The nitrogen loss of egg albumin denatured by heat or acid was less than 0.1 per cent and from 0.1 to 1.0 per cent in the case of alkali denatured egg albumin. Purified denatured egg albumin, denatured by various methods, was studied by means of viscosity, diffusion, electrophoresis, sedimentation (120), and immunochemistry (121). The viscosity of acid denatured egg albumin decreased with time while that of egg albumin denatured by alkali or heat changed very little with time. The electrophoretic mobilities of acid- and heat-denatured egg albumin were similar to that of the main component of egg albumin, the denatured forms being essentially electrophoretically homogeneous. The mobility of alkali denatured egg albumin was considerably lower than that of undenatured egg albumin. Particle weights calculated from diffusion and sedimentation data produced by the same denaturant were found to vary as their final viscosities on aging until the viscosities became constant. Aggregates were found of from 5 to 20 denatured egg albumin molecules. It is apparent from these results that denatured egg albumin is a very unstable substance.

That heat denaturation may involve more than one reaction was shown by a study of the heat denaturation of β-lactoglobulin (122). When heat denaturation of β lactoglobulin was carried out at pH 7.0 in a buffer solution of 0.1 ionic strength, denaturation involved at least two processes which could be followed by means of electrophoretic mobilities. The first process, initiated only above 65°, was accom-

panied by a four-fold increase in particle weight, and an increase in frictional ratio. The second process, which takes place only after the first process has occurred, was accompanied by a marked increase in electrophoretic mobility and particle weight and followed the concentration time characteristics of a second order reaction. The second reaction was repressed at 75° and at 99° it did not take place.

A soluble form of silk fibroin, believed to correspond to a renatured protein, was prepared by dissolving the fibroin in cupriethylenediamine followed by dialysis and neutralization (123). This system is presented as one in which denaturation and its reversal can be studied without any complications due to sulfur linkages or the α- to β-keratin transformation.

The rate of denaturation of human hemoglobin by alkali was found to be greater than the rate of denaturation of ox hemoglobin by alkali (124). The rate of digestion of these undenatured hemoglobins by proteolytic enzymes was the same and it was concluded that denaturation associated with alkali must be different from denaturation associated with proteolysis. Globular proteins are reported to be digested more slowly in the native state than in the denatured state, whereas fibrous proteins are digested at the same rate in the native and denatured state (125).

Further results on the denaturation of serum albumin by sodium dodecyl sulfate indicate that the first step in denaturation consists of stoichiometric binding of the detergent anions by one half of the cationic protein groups (126). When all of the protein cationic groups are combined, the remainder of the molecule becomes disordered. The properties of native and denatured serum globulins denatured by guanidine hydrochloride have been reported (127). Normal globulin is split into components of one half the molecular weight of the native protein $(M = 75,000)$; with the antibody the splitting process stops at the stage of $M = 170,000$. The important observation was made that the irreversibly denatured globulin fraction is serologically active, which indicates that in the sequence of changes brought about by denaturation, serological activity is among the last properties affected. The masking effect of foreign protein during denaturation by urea on serological properties has been demonstrated and is attributed to complex formation between denatured molecules and the grouping concerned in serological reactions (128). The denaturation of egg albumin is reported to involve at least four reactions; the formation of a complex, destruction of biological properties, and the separation of a

soluble and an insoluble portion on the removal of urea (129). Studies
in the temperature range 24.9 to 46.1° gave an activation energy of
27,100 calories per mole for the denaturation of gonadotropin by urea
(130).

The rate of digestion of serum albumin denatured by urea or heat
in the presence and absence of sodium caprylate and related compounds
has been determined (131). It was found that the addition of sodium
caprylate prevents the denaturation by heat or urea as measured by
papain digestion and viscosity increase. Also, the denaturation of
serum albumin by guanidine hydrochloride is prevented by dilute solu-
tions of caprylate (132). Ultrafiltration experiments indicated that
about nine molecules of caprylate combined with each molecule of
albumin. The suggestion was made that caprylate exerts its effect by
combining with the groups in the albumin molecule concerned with
the initial phases of denaturation.

HYDRATION

Most physical measurements on proteins in solution are referred
to the dry weight of the protein. However, it is generally acknowl-
edged that the protein exists in solution as hydrated molecules and
that the behavior of the protein is in many respects dependent on the
water associated with it. For example, measurements on viscosity,
double refraction of flow, or dielectric dispersion can be interpreted
either in terms of hydration or in terms of deviation from spherical
shape (133, 134). Hydration in solution has been defined as the num-
ber of grams of water carried by one gram of protein when it moves
through a solvent (135). As yet there is no unique method of deter-
mining the hydration of proteins in solution.

The value of approximately 0.3 gm. of water per gm. of protein
is considered to be a reasonable value for hydration in solution (134
to 137). This estimate of hydration is based on calculations from di-
electric dispersion, viscosity, and diffusion data as well as from values
for hydration of protein crystals (138). Though these calculations
are probably affected by hydration, it is likely that other factors are
involved. The degree of hydration of viruses has been estimated by
means of sedimentation constants determined in serum albumin solu-
tions extrapolated to the density of no sedimentation (139). The re-
lation between the sedimentation constant of the virus and the density
of serum albumin is essentially constant. Similar experiments in
sucrose solutions gave values for sedimentation which varied with

time, reaching a maximum in about two and one-half hours followed by a decrease. The variation of the sedimentation constant in sucrose is interpreted as a dehydration of the particles by sucrose followed by penetration by sucrose. The densities of influenza viruses A (PR8 Strain) and B (Lee Strain) and swine influenza were estimated from the results of sedimentation in bovine albumin to be 1.104, 1.104, and 1.100 respectively. The true partial specific volumes determined were 0.822, 0.863, and 0.850. From the density values and the partial specific volumes the amounts of water associated with the respective types of virus were 52, 34.5, and 43.3 per cent by volume. These values for the hydration of viruses are of the same order as the values for the hydration of protein crystals obtained by direct measurement. The successful application of this method was favored by the high partial specific volume and high sedimentation constant of the virus. It does not seem probable that the method could be applied to proteins of low molecular weight.

The nature of the hydration of proteins has long been a subject of investigation (140, 141, 142). The curve relating vapor pressure to hydration of protein crystals was found to differ from that of other types of crystalline hydrates (142). The finding that the density of protein crystals varies with the nature of the solvent indicates that substances other than water can penetrate protein crystals (138). It was found that when β-lactoglobulin crystals were suspended in concentrated solutions of ammonium sulfate, the ammonium sulfate penetrated the crystal (143). The important bearing of hydration on the internal structure of the protein molecule as determined by x-ray has been reviewed (144).

The suggestion has frequently been made that the water associated with proteins is bound in more than one way. Thus, in the examination of wet insulin x-ray patterns, gaps were found between the protein molecules. It was suggested that these gaps were filled with liquid of crystallization which would not be carried with the protein in solution. However, on drying of the crystal a greater shift in the position of the molecules than that calculated was noted and the suggestion was made that this excess shrinking might be due to hydration which would persist in solution (144). From the finding that ammonium sulfate diffused into β-lactoglobulin crystals and equalled about 80 per cent of the concentration of solvent ammonium sulfate, the possibility of two kinds of water was suggested (143). Because of technical difficulties the concentration of ammonium sulfate could not

be varied over a sufficient range to determine if this ratio of solvent action of the water in the crystal is constant. However, it has been found that in dilute salt solutions the concentration of ammonium chloride in β-lactoglobulin crystals is even greater than in the solvent (145). A similar result has been found with hemoglobin crystals in dilute salt solutions (138), leaving little support for a classification of the water of protein crystals based on salt penetration. Various methods have been devised for determining what is termed "bound water." Bound water is considered to be water associated with protein which does not behave as a solvent (146, 146a). This and other deviations in behavior, which are often suggested as measures of the state of water in protein systems, are reflections of the properties of the components of complicated systems (147) and are not a measure of hydration.

Factors concerned in protein hydration have been considered in terms of the amino acid composition, the number of polar groups, and molecular packing (140, 141). The comparatively low hydration of silk fibroin is ascribed to a lack of amino acids with large side chains and consequent close molecular packing, and to the absence of a large number of free amino and carboxyl groups (140). The action of formaldehyde on proteins has long been known to reduce the hydration of proteins. This reduction in hydration of proteins by formaldehyde has been correlated with the lysine content (148).

The data derived from the x-ray examination of single protein crystals have also been used to obtain information on protein hydration. From unit cell measurements in the wet and air-dried state, the volume shrinkage of the unit cell may be calculated and if this is attributed to the loss of water, the hydration of the wet crystal may be approximately calculated. Values of 46.6 per cent water for hemoglobin and 40.8 per cent water for lactoglobulin can be obtained from the data given by Crowfoot (144). If the air-dried crystal is assumed to contain 10 per cent water the value is 46 per cent, in good agreement with the directly determined value for lactoglobulin (143). These values are in the range of direct estimates on pepsin (144), edestin (149), and lysozyme hydrochloride (8). Values for hydration of protein crystals obtained by indirect methods (138, 150) are too low because the composition of protein crystals varies with the environment.

If the water content of protein crystals is generally about 45 per cent, it should be possible to use this value to calculate the anhydrous

molecular weight from x-ray data on the wet crystals. In order to make this computation the density must be known. Since most of the reported densities of wet protein crystals are erroneously high (143), an estimated value must be used. The wet density and the water content are related by the equation $\bar{v} = v_p x_p + v_{H_2O} x_{H_2O}$ where $\bar{v}$, v_p, and v_{H_2O} are the specific volume of the crystal and the partial specific volumes of the protein and water respectively. The use of this equation must be based on the assumption that the partial specific volume of the protein is the same in the wet crystal as in dilute solution. This was shown to be true for hemoglobin and edestin by an experiment of Adair & Adair (138) in which the apparent specific volume[1] was determined when the protein was present as a suspension of wet crystals and also when the protein was dissolved.[2]

The density $(1/\bar{v})$ obtained, when values of 46 per cent for the water content $(X_{H_2O} = 0.46)$, of 0.75 for v_p and of 1 for v_{H_2O} are assumed, is 1.15. Using these values and assuming that the air dried crystal contains 10 per cent water the molecular weights calculated from the data (151) are as follows for the wet and dry crystals respectively:[3] ribonuclease (monoclinic) 11,800 and 12,900; lactoglobulin 33,000 and 35,800; methemoglobin 66,000 and 67,000; serum albumin 73,500 and 74,500; bushy stunt virus 11,500,000 and 11,200,000. Lower values for water content must be assumed for insulin and chymotrypsin to obtain agreement.

Proteins show a small but definite decrease in apparent specific volume on going from the dry solid state to aqueous solution (152). This change for egg albumin, serum albumin, and serum globulin is equal to 0.039, 0.036, and 0.037 cc. per gm. respectively (152). For β-lactoglobulin it amounts to 0.043 cc. per gm. [dry density data (143) and apparent specific volume in solution (153)]. Direct determinations of the volume contraction on dissolving casein (142) and gelatin

[1] The partial specific volume is equal to the apparent specific volume if the latter is independent of protein concentration.

[2] This experiment has often been misinterpreted as showing that there is no volume change on dissolving a dry protein. Reference to the conditions of the experiment shows however that the apparent specific volume in solution was compared with the apparent specific volume of the protein in the wet crystal, both being calculated on an anhydrous basis.

[3] The dry density given in the table (151) for bushy stunt virus is the reciprocal of the partial specific volume and is therefore too high for the crystal density. In this case and where no dry densities are given, the value for β-lactoglobulin of 1.26 was used.

(154) are in agreement with these values. These changes are of the same order as those for amino acids (0.030 to 0.041 cc. per gm.) (152). In the case of these compounds the observed decrease in volume can be accounted for by referring it to the electrostriction of the solvent induced by the dipolar structure of the acids. For proteins, however, the use of the maximum estimate for electrostrictive effect will not account for more than 40 per cent of the observed change (152).

The experiment of Adair & Adair (138) referred to above shows that the apparent specific volumes of hemoglobin and edestin are the same in the wet crystals as in dilute solution. Calculations on the composition of β-lactoglobulin crystals show that this is probably true for this protein as well (143). Thus the partial specific volume of the protein is constant over a range of composition from 0 to about 55 per cent protein. The dissolving of proteins is accompanied by a marked evolution of heat (142). The differential heat of swelling curve for casein shows that this occurs almost entirely in regions of low water content and that the heat effects in the range above 40 per cent water are negligible. Thus the contraction in volume (155) and the heat of solution occur entirely in systems of high protein and low water concentration and these phenomena are probably associated with the formation of a hydrated protein structure. As such a system approaches 100 per cent protein the partial specific volume of the protein approaches the specific volume of the dry crystal and the partial specific volume of water will necessarily be lowered. While the partial specific volumes are purely thermodynamic functions and as such are properties of the system, it seems reasonable to relate the lower partial specific volume of water to its combination with the protein to form a tightly held layer of water, presumably by the formation of hydrogen bonds with the polar side chains. This process is accompanied by the evolution of heat. On the addition of more water to the system, the point at which heat effects disappear and the partial specific volumes assume the values characteristic of dilute solution should give an approximation to the water of hydration of the dissolved protein, even though the transition would probably not be abrupt. The hydrated protein and water approach semi-ideal behavior in the range from 0 to 55 per cent protein, probably because the water layer around the protein prevents any further interaction between the components. While any estimate of the water of hydration of a protein from these considerations will have to await investigation of the specific volume relations in these systems with a protein concentration higher than

55 per cent, an upper limit on the hydration is indicated as being the water present in the protein crystal.

The adsorption of water vapor by proteins when calculated according to the theory of Brunauer, Emmett & Teller (BET) for the multi-molecular adsorption of gases (156) results in a value for the completion of the first step in the adsorption process which has been subject to various interpretations. According to the adsorption theory this value should correspond to the formation of a monolayer of the adsorbate on the available surface. However, for egg albumin, a comparison of the adsorption of nitrogen and of water vapor shows that a much larger surface is available to the latter (157). Thus water vapor is able to penetrate portions of the structure not available to nitrogen. Calculation of the surface available to water vapor on the basis of a model of the egg albumin molecule (158), assuming that one layer of water molecules penetrates the surfaces between two adjacent intermolecular polar interfaces of the molecules (but not the assumed intramolecular polar interface), was in agreement with the experimental value.

The value for the extent of the initial adsorption is in good agreement with the value for egg albumin taken from a more extended study (159). For a group of proteins the completion of a second step in the adsorption process was indicated when twice as much water had been adsorbed as corresponded to the first step. This was interpreted as referring to the penetration of a second layer of water between the polar interfaces so that the first layer is no longer shared by adjacent molecules.

In a somewhat different interpretation of the same data (160) a correlation between the water adsorbed in the first step and the total number of polar side chains has been pointed out, one water molecule being adsorbed for each such side chain. In the case of salmin which contains a large preponderance of guanidine side chains, additional layers of water molecules might be added successively, possibly depending on the spatial arrangement of such groups.

These suggestions are consistent with the deduction from x-ray data that all of the water in a crystal of methemoglobin (161) and probably in lactoglobulin and insulin (144) is intermolecular. This would imply that the peptide bonds are not involved as sites of hydration and is borne out by the low water adsorption of unstretched nylon (159). It is probable that the hydrogen bonding capacity of these groups is taken up by intramolecular hydrogen bonding and that they

are thus not available to combine with water. The carbonyl groups equivalent to the proline and hydroxyproline might be an exception to this because these residues in a peptide will lack a hydrogen atom on the peptide nitrogen which is necessary for intramolecular hydrogen bonds (160).

A theoretical treatment of multimolecular adsorption makes it clear that the BET equation can be derived without the restriction that the sites of adsorption be on the surface of the particles (162). This treatment was applied to the adsorption isotherm of wool "reduced" to a common hydrostatic pressure. The rather improbable suggestion was made that the sites of the primary adsorption are the carbonyl groups of the main valence chain.

Many proteins conform to the BET equation up to a relative humidity of about 50 per cent (159). It seems probable that water becomes attached to the protein by the formation of hydrogen bonds with the various polar side chains. This may be equivalent to an interpretation in terms of the area of a polar interface if sufficient regularity of structure is present. However, the plausible assumption that different types of polar groups are characterized by different values for the free energy of combination with water does not lead to an isotherm of the BET type. In any case the amount of water indicated by a BET plot as involved in the primary adsorption is only 12 to 20 per cent of that adsorbed at 95 per cent relative humidity. Thus the considerations discussed above apply to only a portion of the total adsorption. Since most of the water is held in equilibrium with water vapor below saturation it must be considered to be more tightly held than in liquid water. The suggestion that the water adsorbed at high relative humidities is associated with a tendency of the protein to dissolve (159) is not adequate to account for the case of an insoluble protein such as β-lactoglobulin. Crystals of this protein in equilibrium with liquid water contain an amount of water in agreement with that obtained by extrapolating the isotherm to 100 per cent relative humidity (159).

ELECTROPHORESIS

Electrophoresis has proved to be one of the most useful tools in dealing with many aspects of protein chemistry. Valuable information has been obtained by electrophoretic fractionation and the theoretical aspects of this type of work have recently been treated (163). However, the chief utility of the method has been in the analysis of mixtures of proteins, in serving as a guide for chemical methods of

fractionation, and as a criterion of homogeneity. The area analysis of the patterns which is necessary in much of this work cannot, however, be interpreted without reserve as referring to the actual concentrations of the components in a mixture of proteins. Even in cases in which no interaction occurs among the protein components, the changes in conductance across the boundaries introduce a distortion into the relative areas recorded on the pattern. Thus the areas of the peaks will reflect the relative composition of the mixture accurately only if all the components are migrating in an electric field of uniform strength. This condition is not attained in actual work and is only approached at low protein concentrations and relatively high ionic strengths. The concentration and conductance changes across a moving protein boundary have been treated theoretically (164). It was shown that in general, in addition to the gradient for the protein component which disappears in the boundary, there will be a gradient in buffer salt concentration across each boundary and in addition a gradient in protein concentration for each component that appears on both sides of the boundary. The analysis of these effects shows that the conductance below a moving protein boundary is always less than that above it. The superimposed gradients in the ascending boundaries are such that those of the slower components are superimposed by negative gradients in all of the faster components, i.e., the concentration of the component appearing on both sides of the boundary is greater above the boundary than below it. In the descending channel the faster components are superimposed by positive gradients in all of the slower components. Thus in both channels the area on the pattern corresponding to the faster components will be increased at the expense of those due to the slower components. As a result agreement of the area analysis of the ascending and descending patterns is not necessarily proof of the accuracy of the analysis.

Experimental evidence on the influence of ionic strength on the area analysis of electrophoretic patterns was also presented (164). Using swine serum in a phosphate buffer with addition of varying quantities of sodium chloride, the apparent albumin concentration derived from the two patterns became equal when the sodium chloride was between 0.2 and $0.3\,M$. Further increase in salt concentration produced a small change in apparent albumin concentration. The apparent globulin concentrations showed appreciable differences at all ionic strengths. Similar experiments were reported on human plasma in veronal buffers (165). In this work very little difference was found

in the albumin concentration derived from the ascending and descending patterns. The values for the concentration reached a minimum at 0.2 ionic strength and did not change at an ionic strength of 0.3. The globulin components also showed no change above an ionic strength of 0.2. Even smaller differences between the patterns were found for mixtures of bovine serum albumin and horse serum globulin (166). In this case agreement was also obtained between the analysis and the known composition of the mixture.

It seems probable that the more consistent results obtained in these two studies (165, 166) than in the experiments of Svensson (164) were due to the use of phosphate buffer of low buffer capacity by the latter. It has been shown that serum albumin exhibits a marked increase in mobility in phosphate buffers which is related to the increase in phosphate concentration at a constant ionic strength (167) bearing out previous less extensive measurements (168). It is thus evident that erroneous results may be obtained for mobilities measured in phosphate buffers and the need for monovalent buffers covering the range pH 6 to 7 is obvious. The cacodylate buffers which can be used in some cases do not appear to be of general applicability because they sometimes react with the protein. In this regard the buffers of imidazole derivatives (169) should be useful. As a result of improvements (170, 170a, 171) in the synthesis of 4-hydroxy methyl imidazole this substance ($pK = 6.4$) can be synthesized from readily available material. It has been found to be satisfactory for electrophoretic work on casein fractions and should prove to be generally useful. Other imidazole derivatives have values for pK varying from 6.0 to 8.4 (169) so that a wide range could be covered by these buffers.

Electrophoresis has frequently been used as a criterion of the homogeneity of a protein preparation. While the method has a good resolving power in separating components of closely similar properties, there are many cases in which electrophoretically homogeneous preparations have been shown to be mixtures. Extended migration will often resolve boundaries that appear to be uniform in short time experiments, as was found for human serum albumin (172) and myosin (173). The demonstration of homogeneity by electrophoresis is much strengthened if it can be shown to hold over a wide range of pH.

The demonstration of changes in proteins on aging or mild heating has been made in a number of cases. Egg albumin, on standing as a solution or dried powder, shifts to a form having a lower mobility than that of the main component in fresh preparations (174). The aged

preparations are crystallizable. A similar change was found in crystalline horse serum albumin after storage as a sterile solution (175). In this case the change appears to be related to denaturation as somewhat similar alterations were demonstrated to result from heating (176) and exposure to high concentrations of guanidine (177). In the latter case the two components were separated by salt fractionation. One fraction was considered to be "regenerated" albumin whereas the other which was very viscous and insoluble at the isoelectric point appeared to be irreversibly denatured. In other studies of denaturation, additional boundaries were observed in the case of β-lactoglobulin (122), but very little change in the pattern or mobility was found for egg albumin (120). Changes in the mobility and relative distribution of the components took place during the storage of liquid human plasma (178).

Electrophoresis of protein mixtures is often complicated by interaction among the components with the formation of complexes of varying degrees of stability. Except in the case of very stable complexes this is usually reflected in the pattern obtained from the two channels by large differences in the area distribution, mobilities, and even the number of components. Electrophoresis offers one of the few quantitative means of studying such phenomena, but unfortunately patterns of such complexity are often obtained that interpretation is difficult. However, it was possible in a study of the interaction of egg albumin and nucleic acid, to define the behavior sufficiently to explain the observed pattern asymmetries (179). Complicated patterns were obtained in a study of gliadin which showed an extreme dependence on ionic strength and some change with the potential gradient (180). The occurrence of complex formation among the components was confirmed by electrophoretic examination of the separated fast and slow components. Electrophoretic observations on globin, supplemented by ultracentrifugal analysis, indicated that the dependence of the patterns on the pH was a result of the dissociation of the globin molecule into fragments which might recombine in various ways (181). An indication of the formation of unstable complexes between components of peanut protein was found (29, 30). The α- and β-components of casein interact to form complexes which result in only a small degree of pattern asymmetry (182). The electrophoretic pattern of unfractionated casein from human milk is similar to that from bovine casein (183).

The formation of two complexes of definite composition between

horse serum albumin and sodium dodecyl sulfate has been observed in an electrophoretic study of the interaction between these substances (184). At 1° the correspondence between the ascending and descending pattern was close and an analysis of the latter indicated 0.22 and 0.45 gm. detergent combined per gm. of albumin for the complexes 1 and 2 respectively. These observations were supplemented by some at 20°, at which temperature the dodecyl sulfate is soluble and mixtures containing more detergent than that corresponding to complex 2 could be studied. The additional detergent was bound by the albumin although there was some indication of instability of such complexes. The composition of the complexes is equivalent to combination of the detergent with half of the cationic charges on the protein at the pH of the experiments for complex 1 and with all of these charges for complex 2. While it is not possible to distinguish between electrostatic and other types of forces that may be responsible for interaction, on the basis of the influence on the net charge, it has been reasonably assumed that the complex formation is dominated by electrostatic effects. However, it is evident that factors of specific affinity of the anions (van der Waals forces) are involved, at least in stabilizing the complexes, as homologous alkyl sulfates of short chain length did not form complexes of sufficient stability for electrophoretic study (184). This was also shown by the influence on the mobility of human serum albumin of a series of straight chain carboxylic acids (167). The mobility increased at a constant pH and ionic strength in the order of increasing chain length (four to eight carbon atoms) and increased with the concentration of carboxylate ion except at low concentrations of butyrate where a decrease in mobility was evident. In this case the interaction is not complicated by association of the anion into micelles of high molecular weight as with dodecyl sulfate.

Comparative studies have been made on the electrophoretic patterns of plasma (185) and serum (186) from a large number of animal species. Extracts from hair and hair treated with sulfuric acid have been examined electrophoretically (187).

Protein Derivatives and Artificial Fibers

Derivatives.—The preparation of derivatives of native proteins is difficult since the methods used are frequently drastic. The classic studies on artificial conjugate antigens illustrates an important use of protein derivatives (188). The free amino groups of insulin have been

located by means of dinitrofluorobenzene (81). Conjugated proteins with 1,2-benzanthryl isocyanates suitable for carcinogenic studies have been prepared (189). When proteins are treated with phenyl isocyanate in an aqueous alkaline solution the reaction involves the amino, phenol, and thiol groups (190). However, if the reaction of dry protein with isocyanates is carried out in pyridine for twenty-four hours at 70° the amount of isocyanate introduced into the protein is greater than that from aqueous solution (191). From the results of the reaction between phenyl isocyanate and proteins in the presence of anhydrous pyridine it was concluded that the polar groups of the protein, namely, amino, guanido, carboxyl, thiol, phenol, primary amide, and a portion of the aliphatic hydroxyl groups, had reacted with the isocyanate. The protein isocyanate derivatives were found to be resistant to water which is consistent with the finding that the polar groups are blocked by the treatment with isocyanate.

The location of the groups of a protein which combine with formaldehyde in solution is difficult to determine since protein-formaldehyde combinations are somewhat unstable. It is generally conceded that formaldehyde affects primary amino groups of proteins. On the basis of analysis for formaldehyde on a large number of protein-formaldehyde derivatives it was concluded that formaldehyde is combined with the primary amino and primary amide groups of proteins, but not secondary amide and phenolic groups (192). A further indication that formaldehyde combines with primary amide groups, based on the action of formaldehyde on casein and deaminated and deamidated casein, is reported (193).

Artificial protein fibers.—Artificial fibers have been made from the proteins of nuts, beans, eggs, milk, and other nonfibrous proteins. A theory for the production of artificial fibers from nonfibrous proteins has been developed based on x-ray diffraction patterns of α- and β-keratin (194). The folded peptide chains in α-keratin can be converted into the beta form by denaturation and stretching, resulting in a greatly increased tensile strength of the artificial protein fiber (195, 196). Although a variety of globular proteins have been converted into a form giving the β-keratin type of x-ray pattern, the ease of conversion varies with the type of protein (105). Methods for the conversion of casein into fiber have been developed and some of the factors influencing the mechanical properties of the fiber have been evaluated (197, 198, 199). The conversion of vegetable proteins into fibers is being developed on a large scale (200).

Amino Acids and Peptides

Synthesis.—Convenient and economical methods for the synthesis of *dl*-tryptophane have been described (201, 202, 203), which have made this amino acid more available. Indole (201) is converted into gramine (3-dimethylamino methylindole) by condensation with formaldehyde and dimethylamine. Ethyl-α-acetamino-α-carbethoxy-β-(3-indole) propionate is prepared by condensing the methiodide of gramine with the sodium derivative of ethyl acetamino malonate. α-Acetamino-α-carboxy-β-(3-indole) propionic acid is obtained by saponification of the ester. Decarboxylation is accomplished by boiling in water, followed by removal of the acetyl group by alkaline hydrolysis.

The use of ethylacetamidomalonate (171, 204, 205) and acetamidocyanoacetic ester (206) in the synthesis of *dl*-tryptophane, *dl*-histidine, *dl*-methionine, *dl*-valine, *dl*-phenylalanine, *dl*-ornithine, *dl*-norleucine, *dl*-norvaline, *dl*-leucine, and *dl*-glutamic acid has been reported. The synthesis of β-alanine from acrylonitrile has been improved (207, 208). *dl*-Phenylalanine and *dl*-tyrosine were prepared from acrylonitrile and diazonium chlorides (209). Thiobenzoyl derivatives (210) of the amino acids have been prepared by treating thiobenzoylthioglycolic acid with the sodium salt of the amino acid.

Synthetic 3-iodo-*l*-tyrosine and 3-iodo-*dl*-tyrosine (211) were compared with preparations reported to have been isolated from iodinated proteins. The chemical conversion of *dl*-iodotyrosine into thyroxine has been confirmed (212). The transformation is accomplished by incubation of *dl*-iodotyrosine in an alkaline solution. Oxygen is necessary for the transformation and the rate is markedly affected by pH and oxidizing agents.

Cysteyl- and cystyltyrosine and tyrosylcysteine and -cystine and the N-carbobenzoxy derivatives of these peptides were synthesized (213). The cysteine derivatives are more susceptible to hydrolysis by pepsin than are the cystine compounds, which is considered to be related to the fact that denatured proteins with free sulfhydryl groups are more easily digested than the undenatured proteins. The enzymic synthesis of benzoylated amino acids is reported to be improved by the presence of simple sugars (214).

The isolation of *l*-valylglycine from acid hydrolysates of gramicidin was reported (215). Hydrolysis of gramicidin in acid alcohol produced fewer α-amino acids than were expected from the number of

peptide bonds split, assuming that the bonds were split at random (216). Ethanolamine was also found in the hydrolytic products of gramicidin (217). The toxicity of gramicidin was found to be reduced after modification by means of formaldehyde (218). The presence of aspartic acid, valine, leucine, and ornithine equivalent to 5.1, 7.6, 8.2, and 13.1 per cent respectively of the total nitrogen in hydrolyzed tyrocidine was reported (57, 219).

Properties and reactions.—In order to interpret absorption of ultraviolet light by proteins the apparent ionization constants of tyrosine were determined by means of ultraviolet light absorption (220).

The unit cell, probable space group, and the x and z atomic parameters of crystalline metallic salts of glycine and α-aminobutyric acid have been determined (221). The results on glycine confirm previously reported measurements (222).

The reaction of histidine with formaldehyde in neutral and acid solutions at 37° has been determined (223). A stable compound containing one mol of formaldehyde per mol of histidine was isolated. The compound was shown to be $l(-)$1,2,5,6-tetrahydropyrido-3,4-imidazole-6-carboxylic acid. A second mol of formaldehyde combines with this to give a very insoluble methylol derivative. The reaction between solutions of histidine and formaldehyde in the presence of one equivalent of sodium hydroxide has also been followed by polariscopic and hydrogen ion measurements (224). These results were interpreted in terms of two compounds, the first being a methylol type of derivative and the second a combination of two methylol derivatives through a formaldehyde molecule.

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THE CHEMISTRY OF THE STEROIDS

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The authors accepted the invitation to write this article under the assumption that all relevant non-European literature would again be available to them by October 1945. Unfortunately this was not the case. Knowledge of work done overseas had to be drawn chiefly from indirect sources or from a few reprints obtainable by chance. The article therefore is incomplete. In addition such papers as could be reviewed from abstracts have not received due consideration, as this is not possible without knowledge of the original treatises. Restricted as we were in this way, the authors have selected three groups of steroids only and have treated them in three sections. The work covered has been done since the appearance of the last review. These groups include (*a*) sterols since 1944 (1), (*b*) cardiac glycosides and aglycones since 1941 (2), and (*c*) steroid alkaloids since 1939 (3).

STEROLS

Isolation from Natural Sources

The phytosterol fraction of the oil extracted from the seeds of *Calycanthus floridus* has been found to consist mainly of β-sitosterol[1] together with a small amount of α-sitosterol, and possibly sitostanol. *Calycanthus* oil thus resembles cottonseed oil rather than soybean or wheat germ oil, both of which contain γ-sitosterol as the principal steroid component (6). Dicholesteryl ether was isolated from the spinal cord of the ox in amounts corresponding to 1.5 to 2 per cent of the weight of the dry starting material (7).

Constitution, Configuration,[2] and Chemical Transformations

6-Hydroxyl group.—A cholestane-3,6-diol, m.p. 217° (V), was obtained by Windaus (9) by reduction of 6-ketocholesterol (III) with

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[1] For the constitution of β-sitosterol see (4, 5).

[2] Note on nomenclature: the suggestion by Fieser (8) has been adopted, for designating the spatial position of substituents attached to asymmetric carbon atoms of the ring system.

sodium and alcohol. An isomeric diol, m.p. 192° (I), described by
Marker *et al.* (10, 11) is produced from III by catalytic hydrogenation.
In the recent English literature (12) the terms cholestane-3(β),6(α)-
diol for the higher melting product and cholestane-3(β),6(β)-diol for
Marker's compound are used.[3] Plattner & Lang (11) found that in
the diacetate of the higher melting diol (VI) both acetoxy groups are
readily saponified in hot alkaline solution whereas the 6-acetoxy group

I. (R = H) m.p. 192°
   [+ 13 chf]*

II. (R = Ac) m.p. 138°
   [− 23 chf]

III. (R = H) m.p. 148°
    [− 31]

IV. (R = Ac) m.p. 127°
    [− 15 chf]

V. (R = H) m.p. 217°
   [+ 38 chf]

VI. (R = Ac) m.p. 107°
   [+ 39 chf]

VII. (R = H) m.p. 132°
    [+ 10 chf]

VIII. (R = Ac) m.p. 113°
    [− 0.5 chf]

IX. (R = H) m.p. 142°
   [− 46 chf]

X. (R = Ac) m.p. 97°
   [− 46 chf]

XI
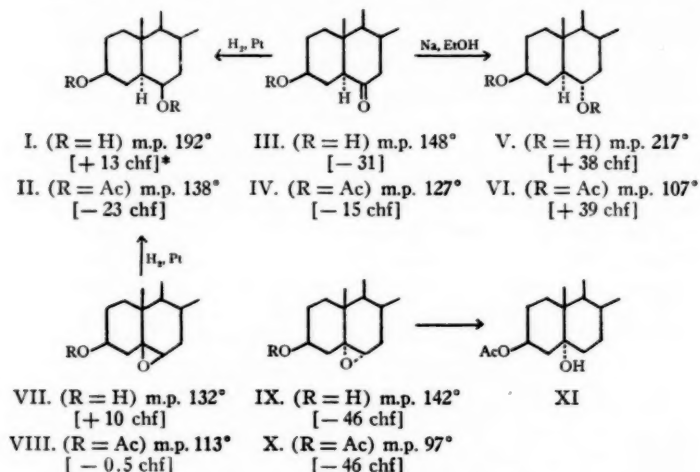
   * The figures in angular brackets give the specific rotations. Chf. is abbreviation for
chloroform.

of the diacetate of the lower melting diol (II) is much more resistant
to saponification. Comparison of stereochemical models [Stuart's type
(13, 14)] of the two diols, (I) and (V), shows that the 6-hydroxyl
group in I should be hindered to a far greater extent than the cor-
responding group in V. This is evidence in support of formula I for
the diol of m.p. 192° and formula V for the diol of m.p. 217° in con-
firmation of the adopted nomenclature. This also firmly establishes
the configuration of some other 6-hydroxy derivatives, the structures
of which are related to I and V and have been partially known for

   [3] Lack of the necessary literature makes it impossible for us to find out
whether this nomenclature is arbitrary or whether it is based on evidence and
is meant to designate the actual spatial position of the 6-hydroxy groups.

some time. For example, Δ^4-cholestene-3,6-diol, m.p. 258° (acetate m.p. 135°), yields the diol I beside other compounds on hydrogenation in glacial acetic acid, and is thus shown to possess formula XII and can therefore be designated as Δ^4-cholestene-3(β),6(β)-diol (15, 16, 17). The isomeric 3(β),6(α)-diol (XIII) has recently been described by Prelog & Tagmann (18). No hydrogenation was carried out, but it has been established that the diol XIII differs only from diol XII in the configuration at position 6. When XII is hydrogenated in alcohol suspension instead of in glacial acetic acid solution (19) the hitherto unknown coprostane-3(β),6(β)-diol (XIV) was obtained

XII. M.p. 258° (di-
acetate m.p. 135°)

XIII. M.p. 175° (di-
acetate m.p. 166°)

XIV. M.p. 198°
[+ 24 chf] (diacetate
m.p. 137° [+ 13 chf])

XV. M.p. 170°
[− 57 chf]

XVI. M.p. 169°
[+ 8 chf]
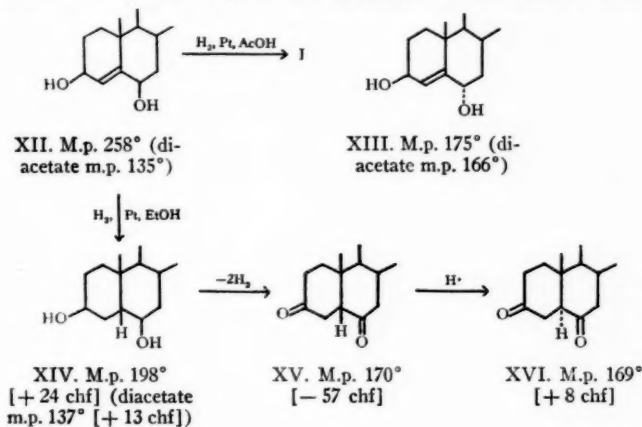
in good yield. This compound is transformed into coprostane-3,6-dione (XV) on oxidation with chromic acid. This dione when heated with acids rearranges to yield the known cholestane-3,6-dione (XVI), which corresponds to the well-known transformation of the dehydro-hyodesoxycholic acid into 3,6-diketoallocholanic acid (20).

Cholesteryl-5:6-oxides.—The two diastereoisomeric cholesteryl oxides have up to now been arbitrarily designated as the α- and β-oxides (21). Since hydrogenation of the acetate of the β-oxide with platinum in glacial acetic acid yields the 3-monoacetate of (I) besides other compounds (22), formula VII must be assigned to the β-oxide and formula IX to the α-oxide. A configurational change at C-5 therefore occurs on hydrogenation. The β-oxide (VII) should be called 3(β)-hydroxy-5:6(β)-oxidocoprostane and the α-oxide (IX), 3(β)-

hydroxy-5:6(α)-oxidocholestane.[4] The arbitrary current nomenclature is thus shown to be correct. By comparing the typical differences of the specific rotations established space formulae may be assigned to the other known oxides of the same type, e.g., the 5:6-oxides of Δ^5-androstene-3(β)-ol-17-one (23), Δ^5-pregnene-3(β)-ol-20-one (24), and Δ^5-pregnene-3(β),21-diol-20-one (25).

5-Hydroxyl group.—Hydrogenation of X with platinum in glacial acetic acid proceeds in quite a different manner from the hydrogenation of VIII and gives good yields of the monoacetate of a diol[5] containing a tertiary bound hydroxyl group (22). Since the bond between the oxygen atom and C-5 is not severed the product formed must be formulated as the 3(β)-acetoxy-5-hydroxycholestane (XI)[6] (11). Similarly 5-hydroxycholestane is formed from cholestane-4:5-oxide and cholestane-5:6-oxide. The 4- and 6-hydroxycholestanes are also formed from the respective oxides (22). However, the authors temporarily refrain from assigning definite space formulae to these oxides, perhaps because of doubt as to their homogeneity. The 5-hydroxyl group in XI is relatively easy to acetylate, which is evidence in favour of a cholestane derivative. In a coprostane derivative steric hindrance due to the methyl group at C-10 should be more marked. The 5-monoacetate (XVII) obtained by partial saponification of the diacetate gives good yields of epicholesteryl acetate (XX) on heating with tosylchloride and pyridine. This remarkable reaction is formulated in analogy to the observations of Winstein *et al.* (27) as follows:

XVII   XVIII   XIX   XX
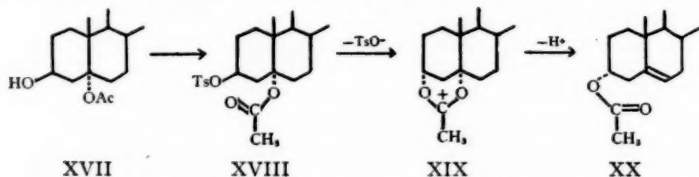
[4] Allocation of an index to the C-5 position can be omitted, since the spatial configuration of the 5-hydroxy group is already designated by the terms coprostane and cholestane.

[5] The same compound has apparently been prepared by Stavely (26) by hydrogenation with palladium in glacial acetic acid. This paper is unfortunately not available to us. Paige (12) reported that hydrogenation of the benzoate of cholesteryl-β-oxide could not be effected with Adams' platinum oxide catalyst, but he obtained small amounts of cholestane-3(β),6(α)-diol (V) both from cholesteryl-α- and β-oxide with sodium and amyl alcohol.

[6] Cf. footnote number 4.

3,5,6-Triols.—The opening of ethylene oxide rings is known to be accompanied by a change of configuration at the C-atom to which the bond with the oxygen atom is ruptured, as has been shown, e.g., in the sugar series (28 to 31). In this way two isomeric *trans*-forms, (XXI) and (XXIII), may result from one oxide (XXII) depending on which link is opened. As a rule, however, one of these forms preponderates or may be produced exclusively. The well-known fission reactions of the two cholesteryl oxides using water, hydrochloric acid, and glacial acetic acid (32, 33) as well as the above mentioned hydrogena-

tion (22, 26) seem to proceed according to this mechanism and may be formulated in the following way (R = H, OH, Cl, OAc):
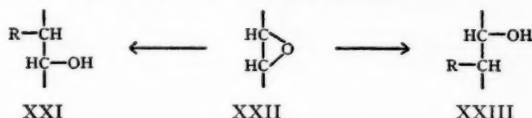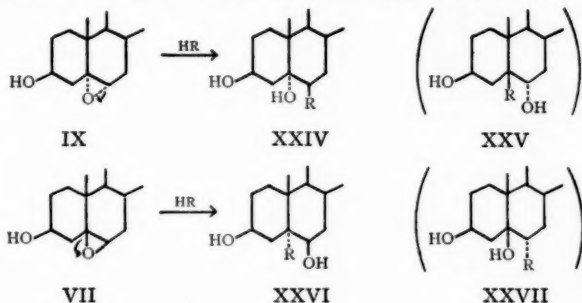
So far the formulae deduced for compounds arising from the fission reactions are in favor of the cholestane derivatives (XXIV) and (XXVI). The equally possible coprostane derivatives (XXV) and (XXVII) are only produced in negligible amounts if at all. Fission of the α-oxide ring, therefore, occurs preferably or only at C-6 and of the β-oxide ring at C-5, as is indicated in the formulae. In accordance with this formulation both oxides (IX) and (VII) (or their respective acetates) yield the same *trans*-triol (XXVIII) (or its 3-monoacetate) when hydrolysed with water in dioxane (11). This formula already had been suggested by Ellis & Petrow (34). The spatial position of the 6-hydroxyl group has been

firmly established by conversion of the compound into cholestane-$3(\beta),6(\beta)$-diol (I). Evidence put forward in favour of a cholestane derivative is not absolutely conclusive [cf. (18), p. 1868, note 6]. In this light the above theoretical considerations are welcome support of formula XXVIII for the "*trans*-triol." On the premise that formula XXVIII correctly represents the "*trans*-triol," Prelog & Tagmann (18) postulate formula XXXII as representing the second known cholestane-3,5,6-triol, the "*cis*-triol," and not formula XXX as had hitherto been assumed (34). In opposition to the views mentioned in the literature (34, 35, 36) XXVIII and XXXII do not yield two different compounds on dehydrogenation with chromic acid but one and the same cholestane-5-ol-3,6-dione (XXXI), m.p. 232°. They there-

XXVIII. M.p. 239°
(diacetate m.p. 165°
[− 47 chf])

XXIX. M.p. 135°
[− 13 chf]

XXX

XXXI. M.p. 232°
[− 13 dioxane]

XXXII. M.p. 236°
(diacetate m.p. 187°)

XXXIII. M.p. 166°
[+ 20 chf]
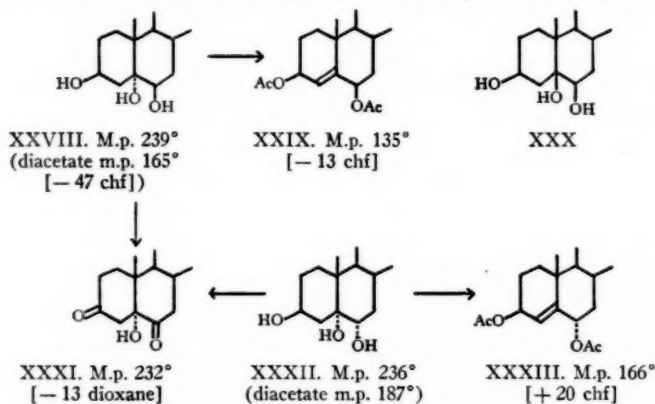
fore possess identical configurations at C-5.[7] The 3,6-diacetate of the *trans*-triol (XXVIII) when treated with thionyl chloride and pyridine yields Δ^4-cholestene-$3(\beta),6(\beta)$-diol diacetate (XXIX) (17), whereas from 3,6-diacetate of the *cis*-triol (XXXII) the isomeric $3(\beta),6(\alpha)$-diol diacetate (XXXIII) is obtained.

"*Hetero-Δ^1-ketones*."—By boiling 2-bromo-3-keto-steroids of the cholestane series with potassium acetate in glacial acetic acid, α,β-unsaturated ketones have been obtained, designated as "hetero-Δ^1-steroid ketones." Their constitutions were elucidated by Butenandt *et al.* (38). Thus 2-bromo-cholestane-3-one (XXXIV) yields Δ^5-cholestene-4-

[7] The isomeric triols from Δ^5-androstene-$3(\beta)$-ol-17-one and Δ^5-pregnene-$3(\beta)$-ol-20-one behave in a like manner (37).

one (XXXV) exclusively. By Clemmensen reduction XXXV is transformed to Δ^5-cholestene (XXXVI). Hydrogenation and subsequent oxidation of XXXV by Oppenauer's method produces the known

XXXIV   XXXV   XXXVI

XXXVII. M.p. 98°   XXXVIII. M.p. 186°
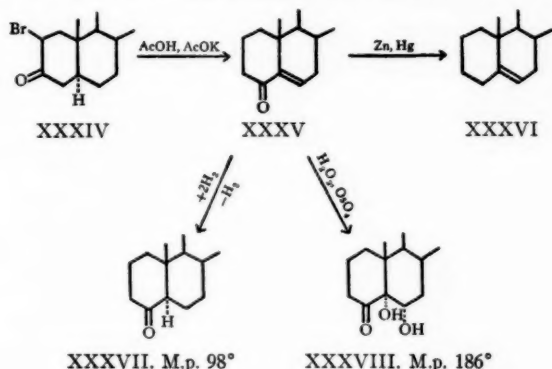
cholestane-4-one (XXXVII), whereas hydroxylation with hydrogen peroxide and osmium tetroxide, or with potassium permanganate, gives cholestane-5,6-diol-4-one (XXXVIII). In this compound only the hydroxyl group at C-6 can be acetylated.

7-Hydroxyl group.—No new experimental evidence bearing on the configuration of the C-7 hydroxyl group has been put forward. However, Wintersteiner & Moore (39) and also Plattner & Heusser (40), from comparison of the specific rotations, called attention to the fact that the arbitrary terms 7(α) and 7(β), as used hitherto, must be erroneous either in the cholestane or in the cholane series. Chenodesoxycholic acid (XXXIX) on treatment with sodium hypobromite furnishes the sodium salt of an acid (XL) which is very easily converted into the lactone acid, "chenodesoxybiliobanic acid" (XLI) (41). On the other hand, ursodesoxycholic acid (XLII) on similar treatment gives an acid (XLIII) which cannot be lactonised (42, 43). It follows, therefore, that in chenodesoxycholic acid the C-7 hydroxyl group possesses the α-configuration. The hitherto applied nomenclature is thus shown to be correct for the cholic acid series, whereas for the cholestane series it is evidently wrong (40). To avoid unnecessary confusion, however, the reviewers suggest waiting for further experimental evidence before modifying the current nomenclature in the cholestane series. By heating either of the two C-7 epimeric 7-

XXXIX. M.p. 140°   XL   XLI
[+ 13 alc]

XLII. M.p. 203°   XLIII
[+ 57 alc]

XLIV   XLV   XLVI
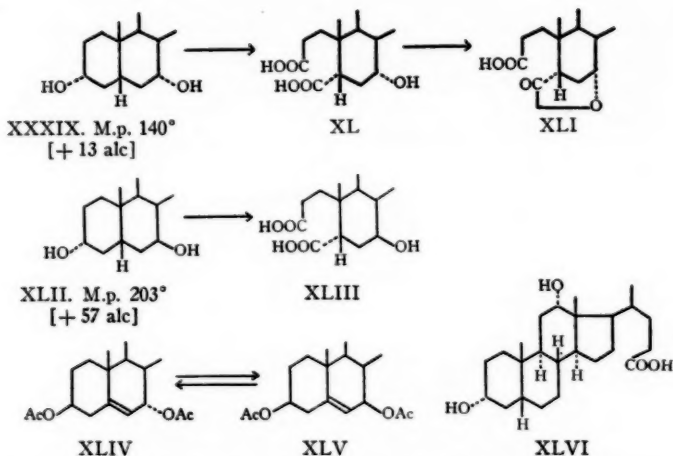
hydroxycholesterol diacetates (XLIV) and (XLV) in glacial acetic acid an equilibrium mixture containing approximately two thirds of XLIV and one third of XLV is obtained. The ease with which epimerisation occurs in acetic acid solution is evidently connected with the allyl grouping present in these compounds (44).

Side chain.[8]—Comparison of the tendencies of bisnordesoxycholic acid and 12-*epi*-bisnordesoxycholic acid to lactonise indicates that the 12-hydroxyl group and the side chain at C-17 in desoxycholic acid (XLVI) are on different sides of the ring system (46), which is contrary to the view held by Giacomello (45). Assuming that the β-position has been attributed correctly to the 12-hydroxyl group by x-ray measurements, Sorkin & Reichstein (46) concluded that the side chain must occupy the α-position. Meanwhile, Gallagher & Long (167) have shown that the opposite is true. The 12-hydroxyl group is in the α-position, the side chain therefore in the β-position. Thus XLVI represents the right formula for desoxycholic acid. Sorkin & Reichstein (168) have also obtained conclusive evidence that this formula is correct.[9] This result also applies to the naturally occuring sterols, bile acids, natural (stabile) progesterone, corticosterone, and "normal"

[8] In order to avoid unnecessary confusion, results obtained in 1946 have been included in this chapter. All compounds of which configuration at C-17 is established are formulated accordingly. These formulae will deviate, therefore, in part from those used in the original papers.

cardiac glycosides, since their side chains have been shown to possess the same configuration as XLVI (46).

Reactions of Δ^4-cholestene-3-one and cholestane-3-one.—Δ^4-Unsaturated 3-keto-steroids are not resistant to heating with lead tetraacetate in glacial acetic acid (47). Δ^4-Cholestene-3-one (XLVII) yields 2-acetoxycholestene-3-one (XLVIII) which on hydrogenation

XLVII   XLVIII   XLIX
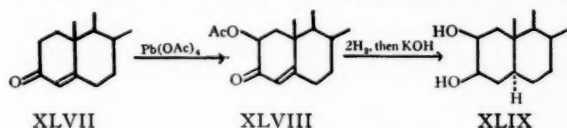
and subsequent saponification unexpectedly yields a cholestane-2,3-diol (XLIX) (48), whereas Δ^4-cholestene-3-one and analogous compounds on hydrogenation preferably give rise to products with coprostane configuration. On treatment of cholestene-3-one (XLVII) with phosphorus oxychloride and pyridine, the pyridinium salt of 3-chloro-

XLVII $\longrightarrow$ L $\longrightarrow$ LI
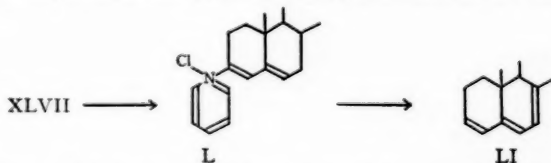
cholestadiene (L) is formed, which on thermal decomposition is transformed into $\Delta^{3,5,7}$-cholestatriene (LI) (49).

Isatin does not react with cholestenone (XLVII). On the other hand cholestane-3-one (LII) reacts with isatin and 5-methylisatin to yield condensation products (LIII) which can be decarboxylated to oxygen-free derivatives (LIV) (50).

LII   LIII. (R = H or CH₃)   LIV
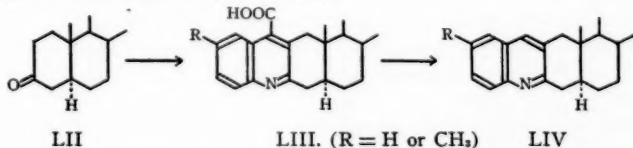
Sterol glycosides.—Plattner & Uffer (51) describe the preparation of glycosides of cholestanol and cholesterol (glucoside, maltoside,

[9] As a consequence of establishing this definite configuration, the hitherto accepted formulae of a whole series of compounds will have to be altered. Thus corticosterone, for example, has β-position at C-11 [see Gallagher & Long (169)]. This cannot, however, be treated here in detail.

cellobioside)[10] in the presence of mercuric acetate or mercury acet-amide. The yields of glycosides were from 30 to 50 per cent.

ANALYSIS

The chromatographic separation of coprosterol from cholesterol has been effected by Christiani (52). Dipole moments of various C-7 substituted steroids have been determined in dioxane solution (53).

METABOLISM

Bloch (54) administered deuteriocholesterol (55) to a woman in the eighth month of pregnancy. From the urine pregnane-3(α),20(α)-diol was isolated as sodium pregnanediol glucuronide and was found to contain significant concentrations of deuterium. The isotope con-centration in pregnanediol was of the same order of magnitude as that of cholesterol circulating in the blood. From these data it can be esti-mated that one half to two thirds of the pregnanediol excreted arose by degradation of cholesterol. These findings suggest that direct con-version of cholesterol to progesterone is a normal process.

Feeding to rats of deuterioacetate leads to the formation of deu-teriocholesterol (56). It is estimated by Bloch & Rittenberg (57) that at least half of the carbon atoms of cholesterol are derived from the acetate. Turfitt (58) found that the microbiological degradation of cholesterol by *Proactinomyces* depends upon the pH value. In a buf-fered solution all the cholesterol completely disappeared after four months. Part of it was oxidized to cholestenone. Other degradation products were isolated but have not yet been identified. Cholestenone is also produced when cholesterol is mixed with garden soil and kept buried for one month. Cholesterol injury in the guinea pig has been discussed by Okey (59).

CARDIAC GLYCOSIDES

ISOLATION FROM NATURAL SOURCES

Strophanthus kombé.—From the seeds of *Strophanthus kombé* cymarin (LVI), k-strophanthin-β (LVII) and k-strophanthoside (LVIII) (60) ["k-strophanthin-γ" (61)] have previously been iso-lated. All these compounds contain strophanthidin (LV) as the agly-cone but differ with regard to their sugar moiety. When the seeds are allowed to stand with water prior to extraction, allocymarin (LVI*a*)

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[10] Probably β-glycosides are formed.

is obtained in addition to cymarin (LVI), the former containing allo-strophanthidin (LVa) as the aglycone. In a recent investigation Katz & Reichstein (62) were able to isolate LVI and LVIa as well as periplocymarin (LX), emicymarin (LXI), their respective allo compounds LXa and LXIa, and a new glycoside, m.p. 217°, with strong cardiac activity from seeds soaked in water. Whether LX and LXI
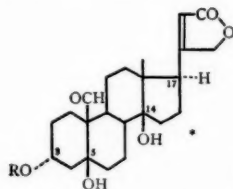
LV. (R = H) strophan-
    thidin
LVI. (R = cymarosido
    residue) cymarin
LVII. (R= glucosido-
    cymarosido residue)
LVIII. (R = diglucosido
    cymarosido residue)
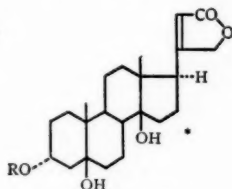
LIX. (R =H)
    periplogenin
LX. (R = cymaro-
    sido residue)
    periplocymarin
LXI. (R = digital-
    osido residue)
    emicymarin
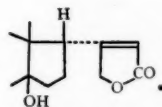
LVa, LVIa, etc.
Allo compounds

* The configuration of the hydroxyl groups at C-3 and C-5 is arbitrarily formulated; the configuration at C-14 and C-17 in the allo compounds is a matter of controversy.

occur as such in the unsoaked seeds or as derivatives of higher sugar content (analogous to LVII and LVIII) has not been ascertained. Girard's reagent is very suitable for the purification of the periplogenin glycosides (LX) and (LXI), but not for the purification of the glyco-sides derived from strophanthidin (LV) and allostrophanthidin (LVa), i.e., LVI, LVIa, etc. This reagent can also be used efficiently in the isolation of sarmentogenin from commercial *"Strophanthus hispidus"* drugs (62) which usually contain seeds of other *Strophanthus* varieties. Positive proof of the constitution of the hitherto un-known alloperiplocymarin (LXa) was given in a later paper (79).

Adonis vernalis.—From the aerial parts of *Adonis vernalis* two crystalline compounds have been isolated besides cymarin. One of these compounds, m.p. 263–265°, has the properties of a glycoside, and its activity in the frog ranks between that of cymarin (LVI) and convallatoxin. The other substance is only slightly active and behaves like an aglycone of the approximate formula $C_{23}H_{32-34}O_7$ (63).

Nerium oleander.—The chemistry, pharmacology, and toxicology

of *Nerium oleander* glycosides which under certain circumstances can replace *Strophanthus* glycosides, have been reviewed by Cortesi (64).

Digitoxin.—For the preparation of pure digitoxin and its conversion to digitoxigenin see the work of Küssner (65).

STRUCTURE AND TRANSFORMATIONS OF NATURAL GLYCOSIDES

Strophanthidol and "k-Strophanthol-γ."—By the reduction of strophanthidin (LV) with amalgamated aluminum or by the Meerwein-Ponndorf method, Rabald & Kraus (61) obtained crystalline strophanthidol (LXII). The heptaacetate of k-strophanthoside (LVIII) (60) similarly furnished crystalline "k-strophanthol-γ" heptaacetate (LXIV). Saponification with barium methylate produced free "k-strophanthol-γ" (LXIII) in crystalline form. Acetylation of (LXIV) gave the octaacetate (LXVI), which on saponification formed the 19-monoacetate (LXV). On hydrolysis with acid LXIII furnished strophanthotriose and strophanthidol (LXII).
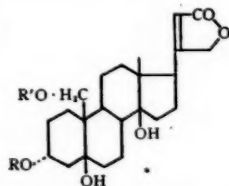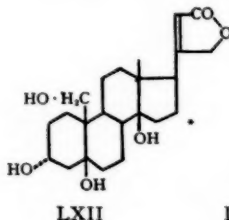
LXII

LXIII. (R′ = H; R = diglucosido-cymarosido residue)
LXIV. (R′ = H; R = heptaacetyl-diglucosido-cymarosido residue)
LXV. (R′ = Ac; R as in LXIII)
LXVI. (R′ = Ac; R as in LXIV)

* In the original paper a β,γ-unsaturated lactone ring is assumed. It is formulated here according to Paist *et al.* (66). The spatial configuration of the hydroxyl groups is arbitrary as in (LV).

Digitoxin.—On degradation of digitoxigenin (LXVII) by a method previously elaborated (69), Hunziker & Reichstein (67, 68) obtained 3(β)-hydroxyetiocholanic acid (LXVIII). Thus in contrast to previous assumptions the C-3 hydroxyl group in digitoxigenin possesses β-configuration. This is remarkable, since the C-3 hydroxyl group in digoxigenin (LXIX), which occurs in the same plant (*Digitalis lanata*), possesses the α-configuration.

LXVII   LXVIII   LXIX
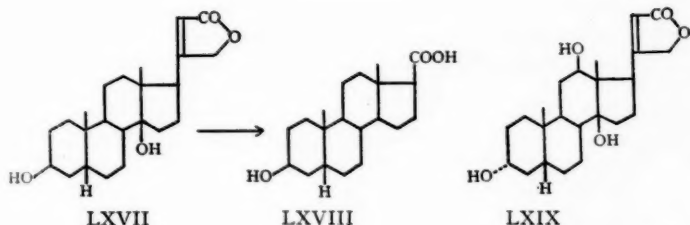
Ouabain (g-strophanthin).—In the aglycone of ouabain six hydroxyl groups are present. Since ouabain on treatment with alkali gives iso-ouabain the presence of a hydroxyl group at C-14 is highly probable. Another hydroxyl group is assumed to be situated at C-19. In previous investigations the trianhydrolactone monoacetate, $C_{24}H_{30}O_4$, m.p. 172°, of Jacobs & Bigelow (70) played an important part. It is obtained from ouabain by the following reactions:

Ouabain $C_{29}H_{44}O_{12}$ $\xrightarrow[35-75°]{Ac_2O + ZnCl_2}$ Anhydro-ouabain heptaacetate* $C_{43}H_{56}O_{19}$ m.p. 283° $\xrightarrow[Pt]{2H_2}$ Dihydro-desoxy-ouabain heptaacetate* $C_{43}H_{60}O_{18}$ m.p. 273° $\xrightarrow[100°]{AcOH + HCl}$ Trianhydro-lactone monoacetate $C_{24}H_{30}O_4$ m.p. 172°
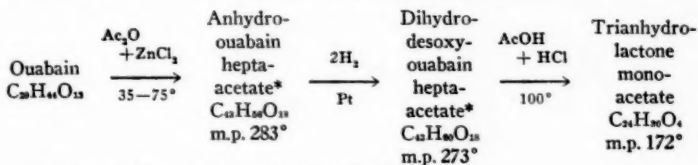
* Analytical figures do not exclude a hexaacetate.

In the third step the rhamnose residue and three acetoxyl groups are lost. One carbon atom is also eliminated as formaldehyde. An analogous lactone has also been obtained from iso-ouabain (71). To explain this reaction Fieser & Newman (72) assumed that ring B had become aromatic (LXX). This was doubted by Tschesche & Haupt (73), and Marker *et al.* (74) believe ring A to be aromatic, since the lactone behaves like estradiol (ring A aromatic) and not like neoergosterol (ring B aromatic) on hydrogenation and in several other reactions. On the basis of Mannich's formula (LXXXVI) (*vide infra*) for ouabaigenin the formation of a lactone (LXXI) would be comprehensible. This formula is also in line with Marker's views, but is opposed by the finding of Tschesche & Haupt that the lactone cannot be dehydrogenated with platinum at 300°. These authors therefore believe that the ring adjacent to the benzene ring is no longer six-membered and has been altered during anhydrisation in an unknown way. Mannich & Siewert (75) opened up a new approach to the

investigation of ouabain and similar glycosides in elaborating an espe-
cially mild method of splitting off the rhamnose moiety which for the
first time permitted the isolation of the unaltered aglycone ouabaigenin

LXX   LXXI
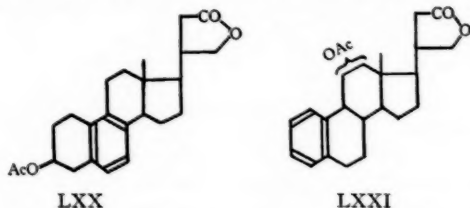
(g-strophanthidin) in good yields. This method is also applicable to
other difficulty hydrolysable glycosides[11] (see below). The reaction is
carried out as follows: ouabain is shaken with acetone containing ap-
proximately 1 per cent concentrated hydrochloric acid (or 0.4 per
cent gaseous hydrochloric acid) at room temperature, whereupon it
dissolves and monoacetone ouabain is formed. The solution is left to
stand from two to three weeks. The sugar residue is probably split off
as the acetone compound and a mixture of monoacetone ouabaigenin
(LXXVI) and an anhydro-ouabaigenin (LXXVIII) results.

The latter compound is also easily obtained on treatment of the
monoacetone ouabaigenin with boiling nitrobenzene. Both substances
on shaking or short heating with very dilute mineral acids are readily
converted into ouabaigenin (LXXIX) which melts at 235–238° when
containing one molecule of water, and at 255–256° when anhydrous.
Its cardiac activity is still considerable, although much less than that
of ouabain. It gives a positive Legal test, can be titrated as a lactone,
and furnishes a dihydroderivative (LXXXII). Of the eight oxygen
atoms two belong to the lactonic group, whilst the other six are proba-
bly present in hydroxyl groups. Four of the hydroxyl groups are easily
acetylated, and the other two are probably tertiary. Dihydro-ouabai-
genin also furnishes a tetraacetate. Since on oxidation of ouabaigenin
with lead tetraacetate only 0.10 to 0.12 moles of the salt are reduced,
it seems that no adjacent hydroxyl groups were present. Thus in

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[11] Intact aglycones have so far been obtained only from the cardiac glycosides,
in which the genin is connected by a glycosidic linkage to a 2-desoxysugar. Only
such glycosides are so easily hydrolysed, that treatment with aqueous or alco-
holic mineral acids of low concentration and short reaction periods suffice to
effect the fission without incurring anhydrisation of the genin.

LXXII
Ouabain hexaacetate
$C_{41}H_{56}O_{18}$, m.p. 290°

LXXIII
Monoacetone ouabain
$C_{32}H_{48}O_{12}$,
m.p. 145–160°

LXXIV
Ouabaigenin diacetate
$C_{27}H_{38}O_{10}$, m.p. 240°

LXXV
Ouabain
$C_{29}H_{44}O_{12}$,
m.p. 187°/241°

LXXVI
Monoacetone
ouabaigenin
$C_{28}H_{38}O_{8}$,
m.p. 200–235°

LXXVII
Monoacetone
ouabaigenin
diacetate
$C_{30}H_{42}O_{10}$, m.p. 268°

LXXVIII
Anhydro-ouabaigenin
$C_{28}H_{32}O_{7}$, m.p. 303°

LXXIX
Ouabaigenin
$C_{23}H_{34}O_{8}$,
m.p. 235°/255°
[+11 W]

LXXX
Ouabaigenin
tetraacetate
$C_{31}H_{42}O_{12}$,
m.p. 282–285°

LXXXI
Anhydro-ouabaigenin
diacetate
$C_{27}H_{36}O_{9}$, m.p. 264°

LXXXII
Dihydro-ouabaigenin
$C_{23}H_{36}O_{8}$, m.p. 261°

LXXXIII
Dihydro-ouabaigenin
tetraacetate
$C_{31}H_{44}O_{12}$, m.p. 264°

W = water

LXXXIV
Dihydro-anhydro-
ouabaigenin
$C_{23}H_{34}O_{7}$,
m.p. 284–287°

LXXXV
Dihydro-ouabaigenin
$C_{23}H_{36}O_{8}$, m.p. 230°

monoacetone ouabaigenin (LXXVI) the isopropylidene residue must
be linked to two α,γ-hydroxyl groups. This is perhaps why the acetone
compound cannot be obtained from free ouabaigenin. The above men-
tioned anhydro-ouabaigenin (LXXVIII) is certainly different in
structure from the known 14-anhydrogenins. On hydrogenation only
one mole of hydrogen is absorbed. It does not react with mono-
perphthalic acid; it is easily hydrated, and gives only a diacetate

(LXXVII). It must therefore be derived from ouabaigenin by oxide formation of two of those hydroxyl groups which can be acetylated. Hydration of dihydro-anhydro-ouabaigenin (LXXXIV) furnishes a dihydro-ouabaigenin (LXXXV), m.p. 230°, which, however, is not identical but isomeric with the compound LXXXII, m.p. 261°, obtained on hydrogenation of ouabaigenin.

Cleavage of ouabain can also be effected with hydrochloric acid in butanone or cyclohexanone as well as in acetone. Analogous products are obtained with these solvents. Mannich & Siewert (75) tentatively suggest the formulae LXXXVI, LXXXVII, and LXXXVIII[12] for ouabain, monoacetone ouabaigenin, and the unstable anhydro-ouabaigenin, m.p. 303°. Contrary to earlier views (72) they see no reason why the sugar moiety should not be linked to the 3-hydroxyl group, since ouabain gives a hexaacetate (LXXII) (three acetoxyl groups in the genin moiety) and ouabaigenin a tetraacetate, i.e., the sugar is evidently linked to a hydroxyl group which can be acetylated.

LXXXVI   LXXXVII   LXXXVIII
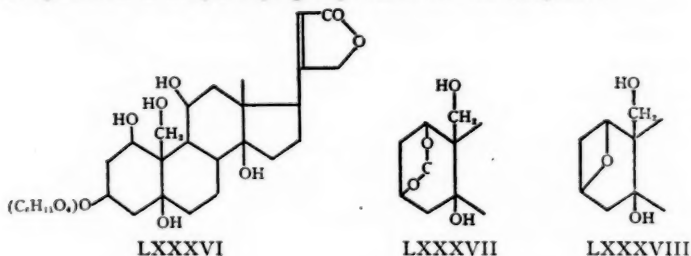
On dehydrogenation of ouabain with platinum and oxygen Mannich & Siewert (76) obtained two isomeric dehydro-ouabains, $C_{29}H_{42}O_{12}$, both of which reduce Fehling's reagent and silver diamine solution, but do not give the fuchsine-sulphurous acid test and do not react with carbonyl reagents. On hydrogenation both isomerides take up two moles of hydrogen. Acid hydrolysis furnishes l-rhamnose and on cleavage with hydrochloric acid and acetone two isomeric anhydrogenins, $C_{23}H_{30}O_7$ are obtained. From this it follows that dehydrogenation of a hydroxyl group in the genin moiety has occurred. The authors ascribe the two C-19 epimeric lactol formulae LXXXIXa and LXXXIXb, analogous to the α- and β-forms in sugars, to the compounds in question.

[12] The authors still formulate the lactone ring with β,γ-double bond.
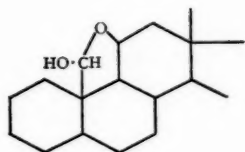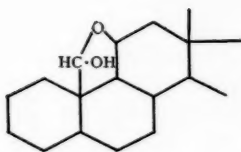
LXXXIX*a*   LXXXIX*b*

Convallatoxin.—With the aid of the method of Mannich & Siewert (75) the cleavage of convallatoxin into *l*-rhamnose and strophanthidin (LV) has been accomplished for the first time with good yields (77). The formula (LV, R = rhamnosido residue) suggested by Fieser & Jacobsen (78) has thus been confirmed.

Emicymarin, alloemicymarin, and alloperiplocymarin.—Application of Mannich's method also enabled the isolation of unaltered periplogenin (LIX) beside digitalose from the cleavage of emicymarin (LXI), thus confirming formula LXI for emicymarin (79). In a like manner alloemicymarin (LXI*a*) gave digitalose and alloperiplogenin (LIX*a*). LIX and LIX*a* are not easily distinguishable from each other. They differ only slightly in their properties, but can be accurately differentiated on conversion to the acetates. Since it has been proved by Lamb & Smith (80) that alloemicymarin (LXI*a*) represents the allomeride of emicymarin (LXI), it follows that alloperiplogenin (LIX*a*) derived from the former must be the allo form of LIX. Alloperiplocymarin (LX*a*), previously isolated from the seeds of *Strophanthus kombé* (see above), yields cymarose and an aglycone on mild hydrolysis. This aglycone was shown to be identical with alloperiplogenin (LIX*a*) obtained from LXI*a*. Its constitution is therefore established. Alloperiplocymarin (LX*a*) and periplocymarin (LX) cannot be clearly distinguished even as acetates. Differentiation is best effected by hydrolysis and subsequent acetylation of the resulting genins. The maxima in the ultraviolet absorption spectra of the acetates of alloemicymarin and alloperiplogenin are practically identical with those obtained from the biologically active forms. The double bond, therefore, is also located in the α,β-position (62).

Sarmentogenin.—Reinvestigation (62) has confirmed that sarmentogenin and digoxigenin (LXIX) give rise to two isomeric ketones when dehydrogenated with chromic acid under identical conditions. Thus sarmentogenin like LXIX contains two secondary hydroxyl groups. It must, however, differ from LXIX apart from epimerism.

For digoxigenone (XC) obtained from (LXIX) the positions 3 and 12 for the carbonyl groups are established. According to previous investigators the ketonic groups in sarmentogenone (XCII) might occupy the C-3 and C-11 positions (XCI). Comparison of optical rotations, however, does not favour this view, since the values for the molecular rotations in the two diketonic compounds differ to a greater degree than is usually the case when two compounds differ only in that one has the ketone group at C-11 and the other at C-12 (62).
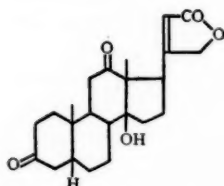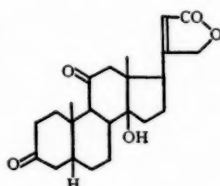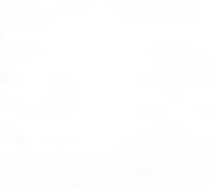
XC. Digoxigenone   XCI   XCII. Sarmentogenone
m.p. 254–260° [+ 121]   m.p. 235–237° [+ 14]

Scilliroside.—For the scilliroside isolated from the red variety of *Scilla maritima* (81), which is particularly toxic to rats, Stoll *et al.* (82) suggested formula XCVII. This formula has not been proved in detail. It is based chiefly on the following facts. One of the two hydroxyl groups inert to acetylation, for which 12-position is assumed, is secondary, since it can be easily dehydrogenated with chromic acid to give a ketone. It is remarkable that this dehydrogenation also occurs with lead tetraacetate under conditions so mild that reaction occurs not only with the tetraacetate (XCVIII) but also with free scilliroside (XCVII) without attack of the sugar moiety. The ultraviolet absorption spectrum shows that the ketone group and the double bond (assumed to be between C-8 and C-9) are not conjugated. This double bond cannot be hydrogenated in XCVIII but in the ketone (C) hydrogenation occurs. This is regarded as evidence against position C-16 for the secondary hydroxyl group. Similarly the $\Delta^{8,9}$-double bond can be hydrogenated after removal of the other hydroxyl group, assumed to be at C-14, by heating with sulphuric acid. Elimination of the C-14 hydroxyl group occurs with still greater ease in the ketone (C) to yield a substance, for which structure XCVI is suggested. Apart from the doubly unsaturated lactone ring this substance contains another system with strong ultraviolet absorption. From the

reactions mentioned it follows that the tertiary hydroxyl group, the secondary hydroxyl group (inert to acetylation), and also the double bond must be located in near-by positions. If the secondary hydroxyl group were assumed to be at C-15, oxidation should lead to cleavage of the linkage between C-14 and C-15.

XCIII   Pb(OAc)₄   XCIV   H₂, Pt   XCV   XCVI
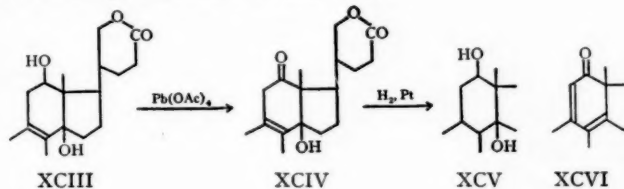
On formation of the *iso*-compound from XCVIII, ring closure does not take place at C-14 as was formerly assumed but at C-12 since the acetylated methylester (CI) cannot be dehydrogenated with chromic acid. Owing to the fact that the ketone (C) can also be isomerised, formula CIII has been suggested to describe the corresponding acetylated methyl ester of the *iso*-acid.

As has been previously shown (83), hydrogenation of XCVIII using platinum yields an amorphous mixture, whereas with palladium exactly three moles of hydrogen are taken up and one acetoxy group is lost, giving a crystalline product containing a saturated lactone ring (XCIII). This was shown to be a mixture of two isomerides, which probably differ in their configuration at C-20. The main reaction product, m.p. 240°, was dehydrogenated with lead tetraacetate to yield the ketone (XCIV), which on further hydrogenation with platinum absorbed two moles of hydrogen and furnished the completely saturated lactone (XCV). XCVIII as well as XCIII consumed one mole of oxygen on treatment with perbenzoic acid and yielded crystalline oxides. The methyl ester CI gave the ester CII on hydrogenation with platinum. After saponification of the methyl ester group and the four acetyl groups of the sugar moiety the product was heated with acid, which treatment resulted in splitting off the glucose residue as well as the removal of the C-14 hydroxyl group as water. Judging by the ultraviolet absorption spectrum the acid (CIV) or (CV) thus obtained contains a conjugated system of two double bonds situated in two different rings. Both of these are hydrogenated with platinum, producing a saturated acid (CVI).

Hellebrin.—For the crystalline glycoside $C_{36}H_{52}O_{15}$ or $C_{36}H_{52}O_{16}$

previously isolated from Radix Hellebori nigri, Karrer (84) suggested formula CVIII. It possesses an activity of 2500 to 3200 frog doses per mg. and is therefore more active than ouabain. Investigation was rendered difficult by the fact that hydrolytic cleavage of the glycosidic

XCVII. (R = H)
XCVIII. (R = Ac)

XCIX. (R = H)
C. (R = Ac)

CI

CII

CIII

CIV

CV

CVI

CVII

CVIII

CIX
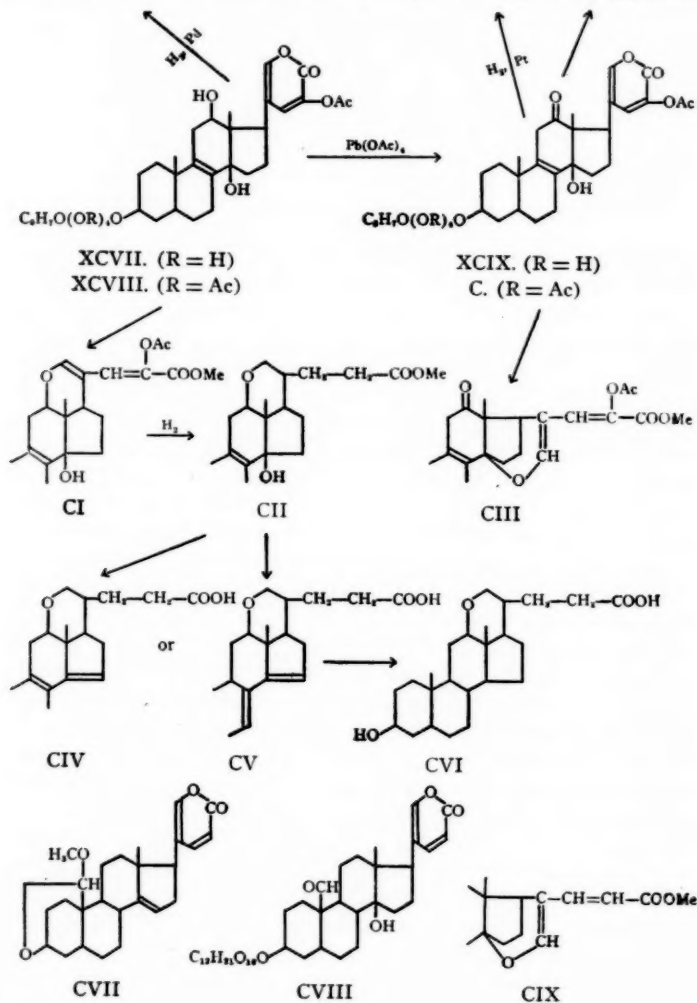
linkage requires such drastic treatment that the aglycone is altered. Formula CVIII is mainly based on the following observations. Hellebrin reveals selective absorption in the ultraviolet range with a maximum at 300 mμ exactly like scillaren A (85). On treatment with hot methyl alcoholic potassium hydroxide it is converted to the methyl ester of isohellebrinic acid (CIX). Acetylation furnishes a heptaacetate. On heating with aqueous sulphuric acid two molecules of glucose and amorphous derivatives of the aglycone are formed. Hydrochloric acid in methanol yields beside α-methyl-*d*-glucoside a crystalline compound $C_{25}H_{32}O_4$, for which formula CVII has been suggested.

Diginin.—This inactive glycoside $C_{28}H_{40}O_7$ isolated by Karrer (86) from *Digitalis purpurea* is split into diginigenin $C_{21}H_{28}O_4$ and diginose $C_7H_{14}O_4$ on mild hydrolysis. The sugar is isomeric with cymarose but differs from the latter at least in the configuration of the methoxyl group at C-3 (87). Earlier investigations (88) disclosed diginigenin to contain one carbon to carbon C-C double bond which is easily hydrogenated, and a very reactive carbonyl group. The two remaining oxygen atoms must either be present as ether linkages or else one must belong to a very inert keto group. Further degradation experiments by Shoppee (89, 90) revealed the possible presence of a tetrahydrofuranone ring (CX). By applying a combination of
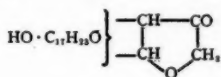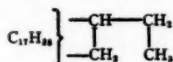
CX   CXI

different reduction and oxidation procedures this compound was converted to the saturated, crystalline hydrocarbon "diginane," $C_{21}H_{36}$, m.p. 75–77°, which should thus contain four rings. It is not identical, however, with any known hydrocarbon compound of this composition. It has not been established whether it represents the basic hydrocarbon skeleton of diginigenin according to formula CXI or whether some rearrangement has occurred in the reaction leading to its formation. Therefore it is not yet established that diginigenin is a steroid.

SYNTHETIC GLYCOSIDES FROM NATURAL AGLYCONES; RELATIONS BETWEEN CONSTITUTION AND DIGITALIS-LIKE ACTION

Uhle & Elderfield (91) synthesised the acetates and the free β-glycosides of strophanthidin with *l*-arabinose, *d*-xylose, *d*-glucose, and *d*-galactose. All four acetylglycosides were obtained in crystalline

form from strophanthidin and the acetobromo sugar in dioxane in the presence of silver carbonate and anhydrous magnesium sulphate in yields ranging from 7 to 31 per cent. As this method is a variation of the classical procedure of Koenigs & Knorr which is known to lead to β-glycosides the β-configuration is attributed to the new glycosides. Deacetylation was carried out with barium methoxide in methanol. The free glycosides of *l*-arabinose, *d*-xylose, and *d*-glucose were obtained in crystalline form as hydrates; the derivative of *d*-galactose did not crystallise. The seven crystalline substances together with strophanthidin acetate (92) have been assayed by Chen & Elderfield (93) in cats and frogs. Results are summarized in Table I, together with previous data (94, cf. 95) for strophanthidin, cymarin, and convallatoxin and for ouabain.

TABLE I

BIOLOGICAL ASSAY OF SYNTHETIC GLYCOSIDES AND RELATED COMPOUNDS

Compound	Assay in Cats: Mean Lethal Dose ± Standard Error (μg./kg. Cat)	Assay in Frogs: Mean Systolic Dose ± Standard Error (μg./gm. Frog)
Strophanthidin	306.2 ± 38.7	2.71 ± 0.49
Strophanthidin acetate	186.6 ± 24.6	2.19 ± 0.13
Strophanthidin-β-tetraacetyl-*d*-glucoside	1166 ± 125	18.77 ± 3.07
Strophanthidin-β-*d*-glucoside	91.3 ± 2.46	0.583 ± 0.04
Strophanthidin-β-triacetyl-*d*-xyloside	591.6 ± 70.4	8.07 ± 1.35
Strophanthidin-β-*d*-xyloside	109.5 ± 4.39	0.64 ± 0.04
Strophanthidin-β-triacetyl-*l*-arabinoside	1230 ± 136.6	6.33 ± 0.38
Strophanthidin-β-*l*-arabinoside	94.5 ± 2.95	0.308 ± 0.03
Strophanthidin-β-tetraacetyl-*d*-galactoside	1692 ± 168	11.29 ± 1.85
Cymarin	110.1 ± 3.75	0.60 ± 0.006
Convallatoxin	79.0 ± 3.1	0.21 ± ?
Ouabain	116.0 ± 2.7	0.828 ± 0.02

Thus the three free synthetic glycosides proved to be very active, two of them even considerably more so than cymarin, the natural glycoside from which strophanthidin is originally obtained. On the other hand the acetyl glycosides were much less potent even than strophanthidin. It should be noted that strophanthidin acetate is more potent than strophanthidin, confirmatory of Neumann's findings (92). Esterification of digitaloid aglycones often increases the cardiac activity,[13] but

[13] Aminoacid esters of strophanthidin, digitoxigenin, gitoxigenin and gitoxin possessing high activity were described by Küssner *et al.* (96). Assays of various esters of strophanthidin in cats were reported by Steldt *et al* (97, cf. 95).

as demonstrated for the acetylation of cinobufagin and marinobufagin, esterification may also reduce it (98). In the glycosides single acetyl groups may produce a measurable increase of potency—the opposite effect from that produced by complete acetylation of the sugar portion of the molecule in the four compounds mentioned above. Thus oleandrin which contains one acetyl group in the steroid ring system is more potent than desacetyl oleandrin (99, 100), and the digilanides A and B which contain one acetyl group in the sugar moiety were shown to be more potent, at least in cats, than desacetyl digilanides A and B, respectively (101).

PARTIAL SYNTHESIS OF DIGITALIS-LIKE AGLYCONES

Numerous compounds of the type CXVI which are mostly designated as etiocholanyl-butenolides or 21-hydroxynorcholenic acid lactones have been prepared according to two different methods. The first and better of the two methods utilises 21-acetoxy- or 21-benzoxy-pregnane-20-one-derivatives (CXIII) or (CXIV) as starting material which can be prepared in the known way from etio acids (CXII) and the intermediate 21-diazoketones (102) or from methyl ketones (CXVII) with lead tetraacetate (103). Acetoxylation with lead tetraacetate affords good yields provided that no groups readily affected by this reagent like reactive keto groups or unprotected hydroxyl groups (47, 104) are present.

CXII   CXIII. (R = MeCO)   CXV   CXVI
         CXIV. (R = PhCO)

CXVII   CXVIII   CXIX   CXX
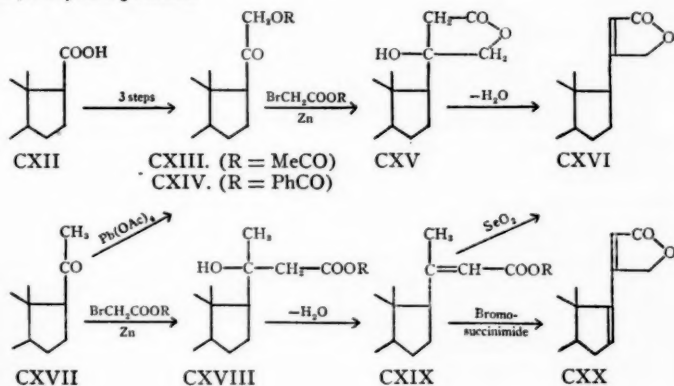
On reaction of CXIII or CXIV with ethyl bromoacetate and zinc according to Reformatsky the hydroxylactone (CXV) is obtained

after loss of ethyl acetate or ethyl benzoate. Splitting off water from the hydroxylactone (CXV) then gives the unsaturated lactone (CXVI) (105, 106, 107). The second method involves interaction of the methylketone (CXVII) with ethyl bromoacetate and zinc. The hydroxyester (CXVIII) thus obtained is converted into the $\Delta^{20,22}$ norcholenic acid ester (CXIX) by dehydration. Heating with selenium dioxide furnishes the lactone (CXVI). So far, however, yields obtainable with the second method are not satisfactory (108, 109). Experiments utilising Ziegler's N-bromosuccinimide method (110) instead of selenium dioxide for the conversion of CXIX into CXVI, yielded only small amounts of the doubly unsaturated lactone (CXX) (111) instead of CXVI. Plattner & Heusser (112) made a further attempt to synthesise the lactone ring. The diazoketone (CXXI), an intermediate on conversion of CXII into CXIII, yielded the bromi-

nated ester (CXXII) on heating with bromoacetic acid. When heated with zinc this bromoester could be converted into the lactone (CXVI), but only if ethyl bromoacetate were added simultaneously. There is, therefore, uncertainty as to whether intramolecular ring closure has occurred or whether the reaction follows the same course as in the first method.
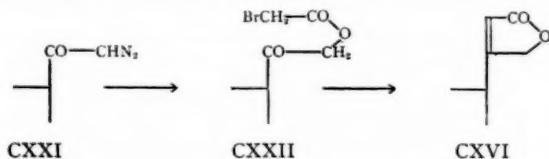
Apart from various model β-substituted butenolides (113 to 118), the following compounds of the CXVI type have been prepared using these methods, but especially the first one.[14] Δ^5-3(β)-hydroxyetiocholenyl-butenolide (105, 106, 112), 3(α)-hydroxyetiocholanyl-butenolide (120), 3(β)-hydroxyalloetiocholanyl-butenolide (108, 121, 122), etiocholanyl- and 3(β)-hydroxyetiocholanyl-butenolide(14-desoxydigitoxigenin) (107), Δ^5-3(β)-hydroxynorcholenyl - butenolide (112, 123), norcholanyl-butenolide (124), 3(α)-hydroxyalloetiocholanyl- and alloetiocholanyl-butenolide (122), 3(α),7(α), 12(β)-trihydroxynorcholanyl-butenolide (124, 125), 3(α),12(β)-dihydroxyetiocholanyl-butenolide (109), Δ^5-3(β)-hydroxyetiocholenylmethyl- and

[14] This list is perhaps incomplete. Occasionally side reactions occurred. Thus 12-hydroxy-derivatives gave rise to (23→12)-lactones (119).

3(β)-hydroxyalloetiocholanlylmethyl-butenolide (126), and Δ²-allo-etiocholenyl-butenolide (127). Synthesis of two lactones containing a methyl group in the α-position to the carbonyl group has also been achieved (128). With some of these lactones further transformations were effected (127, 129). The first lactone thus prepared, Δ⁵-3(β)-hydroxyetiocholenyl-butenolide, was convertible to a compound prob-ably identical with a substance obtained by conversion of uzarigenin (106). An additional correlation of synthetic material with a natu-rally occurring glycoside has been accomplished by Plattner et al. (130). 3(α),12(β)-Dihydroxyetiocholanyl-butenolide (CXXIV) yielded two isomeric dihydrolactones (CXXIII) and (CXXV) on hydrogenation.
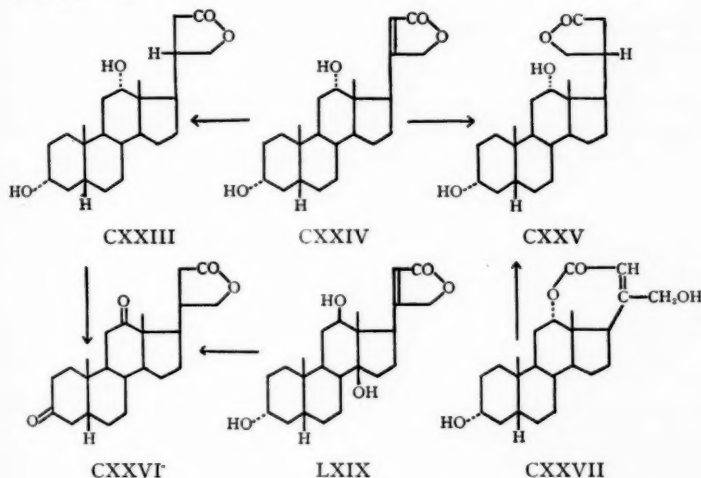
Oxidation of CXXIII with chromic acid produced a diketolactone (CXXVI), which is identical with tetrahydroanhydrodigoxigenone obtained from digoxigenin (LXIX). The other lactone (CXXV) proved to be identical with a compound obtained from CXXVII after hydrogenation and rearrangement (119). All butenolides described give the well-known Legal test,[15] and those examined spectroscopically reveal selective absorption in the ultraviolet with a high maximum

[15] Paist et al. (131) describe an especially sensitive variation in which potas-sium ferricyanide is used instead of sodium nitroprusside.

(log ε = approx. 4.2) at approximately 215 to 220 mμ, in close agreement with the values obtained with natural lactones of the digitalis and strophanthus types (131). They differ, however, from natural aglycones in lacking a hydroxyl group at C-14. Since, so far as is known, cardiac activity of natural glycosides and aglycones is dependent upon the presence of the C-14 hydroxyl group[16] considerable activity is hardly to be expected from any of these. However, lack of reports on this subject precludes any precise statement. Glycosides have been prepared from two lactones, i.e., Δ^5-3(β)-hydroxyetiocholenyl-butenolide and Δ^5-3(β)-hydroxynorcholenyl-butenolide (51, 132). From the former also the maltoside has been obtained (132), which in water is soluble to the extent of 0.02 and 0.1 per cent at 20° and 95° respectively.

STEROID ALKALOIDS

Alkaloids have been extracted from a great number of *Solanum* species (3, 133), but only a few bases have been closely examined and their purity ascertained. The steroid alkaloids belong to the small group of glycoalkaloids; the ones best known are listed in Table II.

TABLE II
GLYCOALKALOIDS

Plant Origin	Glycoalkaloid	Formula	Aglycone	Formula
S. tuberosum (potato), *nigrum* (woody-night-shade), *lycopersicum* (tomato)	Solanine	$C_{45}H_{73}O_{15}N$ (134)	Solanidine	$C_{27}H_{43}ON$
S. sodomaeum, aviculare, xanthocarpum	Solasonine	$C_{45}H_{73}O_{16}N$ (135)	Solasodine	$C_{27}H_{43}O_2N$
S. auriculatum	Solauricine	$C_{45}H_{73}O_{16}N$ (136)	Solauricidine	$C_{27}H_{43}O_2N$
S. angustifolium	Solangustine	$C_{33}H_{53}O_7N$ (137)	Solangustidine	$C_{27}H_{43}O_2N$
S. pseudocapsicum	?	?	Solanocapsine	$C_{26}H_{44}O_2N_2$ (138)
			Solanocapsidine	$C_{26}H_{44}O_4N_2$ (138)

[16] This applies only to this group of substances, and is not altogether beyond doubt. On the other hand compounds with digitaloid activity are known, which are not steroids, e.g., the alkaloids from *Erythrophleum*.

Since the whole field of the steroid alkaloids has been reviewed in Henry's *Plant Alkaloids* (3), only additional data are treated in this report. No further work has been done on solangustine (137) and solanocapsine (138).

Rochelmeyer (139) and Briggs (134, 135, 140) have found that solasonine (formerly called solanine-s) and its aglycone have the above mentioned formulae and not $C_{54}H_{98}O_{19}N_2 \cdot H_2O$ and $C_{18}H_{31}ON$ respectively, as discussed by Oddo [cf. (3)]. Hereby solasonine and its aglycone were brought into line with other steroid alkaloids, the aglycones of which now are all shown to have twenty-seven carbon atoms [solanocapsine may have twenty-six or twenty-seven (141)]. The base extracted from *Solanum aviculare* has been identified as solasonine (140). Contrary to the idea expressed earlier (142) solauricine extracted from *Solanum auriculatum* is not identical with solasonine. Although these glycosides as well as their aglycones are very difficult to distinguish, it has been found that solasonine and solauricine are individual isomeric bases (136).

Steroid skeleton.—The presence of a steroid skeleton in solanidine, solasodine, and solanocapsidine was proved by selenium dehydrogenation, whereby γ-methyl-1,2-cyclopentenophenanthrene (CXXVIII) was obtained (3).

Solanidine
Solasodine } $\xrightarrow[300°]{Se}$
Solanocapsidine

CXXVIII
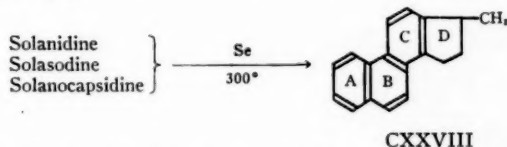
Double bond and hydroxyl groups.—The presence of a double bond is not reported for solangustidine and is denied for solanocapsine, whereas all other alkaloids contain at least one double bond in 5,6-position. Like most steroids, the *Solanum* alkaloids contain one secondary hydroxyl group at C-3 (with the exception of solanocapsine), and it is also at this carbon atom that the sugar moiety must be linked to the steroid skeleton. The partial formula CXXX, in which rings A and B are identical with those of cholesterol, is thus attributed to these alkaloids. The structure of this part of the molecule must be regarded as well established since all characteristic reactions of cholesterol pertaining to rings A and B are also given by these alkaloids. The most important of these reactions is the formation of sparingly soluble digitonides. Such insoluble digitonides have been observed

with solanidine (3), solasodine (143), and solauricidine (136), but
not with their acetates. No precipitate occurs, however, with solano-
capsine, in which the hydroxyl group is believed not to be in position 3.

CXXIX   CXXX. (R = H)   CXXXII   CXXXIII
           CXXXI. (R = Ac)

CXXXIV   CXXXV   CXXXVI   CXXXVII

CXXXVIII. (R = H)   CXL   CXLI   CXLII
   CXXXIX.
   (R = PhCO −)
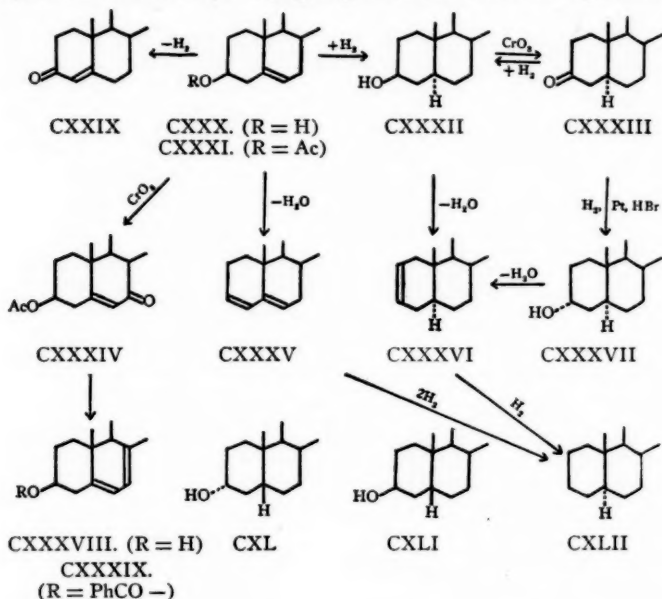
The α,β-unsaturated ketones, solanidenone (144) and solasodenone
(CXXIX) (139), possessing ultraviolet absorption spectra identical
with cholestenone were obtained by dehydrogenation of solanidine and
solasodine (CXXX), respectively, with copper (134, 144, 145), or by
Oppenauer's method (139, 146, 147). Oxidation of solanidine acetate
(CXXXI) with chromic acid yielded 7-ketosolanidine acetate
(CXXXIV). This compound by a series of reactions corresponding to
the transformation of cholesterol into 7-dehydrocholesterol could be
converted into 7-dehydrosolanidine benzoate (CXXXIX) possessing
the same ultraviolet absorption spectrum as ergosterol (148). The
saturated solanidane-3(β)-ol (CXXXII) (3) can be dehydrogenated
to solanidanone (CXXXIII) by the Oppenauer procedure (146, 147).
If this compound is hydrogenated in the presence of hydrobromic acid
solanidane-3(α)-ol (CXXXVII) (m.p. 212° and not 190° as reported

earlier) is obtained, which unlike CXXXII is no longer precipitable with digitonin (146, 147). Two new stereoisomeric compounds allo-solanidane-3(α)-ol[17] (CXL) and allosolanidane-3(β)-ol[17] (CXLI) have been prepared by Prelog & Szpilfogel (147) from CXXIX with platinized Raney nickel. The four isomers CXXXII, CXXXVII, CXL, and CXLI correspond to cholestane-3(β)- and 3(α)-ol and to coprostane-3(α)- and 3(β)-ol. If the keto group of CXXIX only is reduced by the Meerwein-Ponndorf method Δ^4-solanidene-3(α)-ol (CXLIII) and Δ^4-solanidene-3(β)-ol (CXLIV) are obtained (145, 146), CXLIV, but not CXLIII, being precipitable with digitonin.

CXLIII   CXXIX   CXLIV   CXLV
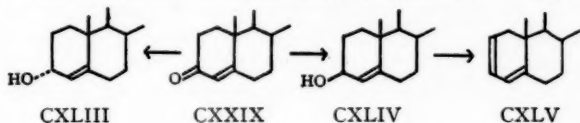
Solanidiene [solanthrene of earlier papers, cf. (3)] and solasodiene (134, 139, 143), both obtainable from the corresponding alkaloids (CXXX) by dehydration, possess the partial formula CXXXV. This is indicated by the facts that they do not react with maleic anhydride and that they have ultraviolet absorption spectra similar to $\Delta^{3,5}$-cholestadiene. An isomeric diene (CXLV) possessing an ultraviolet absorption spectrum similar to $\Delta^{2,4}$-cholestadiene was formed when the benzoate of Δ^4-solanidene-3-(β)-ol (CXLIV) was heated with dimethylaniline (146). The saturated base solanidane (CXLII), $C_{27}H_{45}N$, was obtained by hydrogenation of CXXXV or CXXXVI (147); the latter has been prepared from CXXXII and CXXXVII with boron trioxide. Analogous dehydration of CXL yielded Δ^2-allosolanidene[17] (CXLVI), which on hydrogenation gave allosolanidane[17] (CXLVII), isomeric with CXLII (147).

CXL   CXLVI   CXLVII
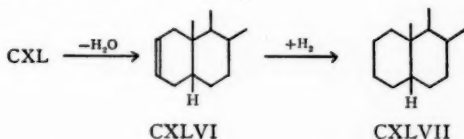
Sugar-moiety of the steroid alkaloids.—The trisaccharide rhamnosido-galactosido-glucose has not hitherto been found in other natural

[17] Nomenclature as regards C-5 is the inverse of that used in the bile acid series.

products (134). Contrary to older views [cf. (3), p. 647], in solasonine only one aglycone is joined to the trisaccharide (134). This also applies to solauricine (149). Solangustine contains only one glucose molecule (137) and solanocapsine and solanocapsidine apparently contain no sugar (138).

Basic part of the aglycone molecule.—No definite proof for the structure of the basic part of the *Solanum* alkaloids has yet been put forward. Except for solanocapsine (138) and perhaps for solangustidine (137), the nitrogen in all alkaloids is tertiary. Hofmann degradations and the reactions with cyanogenbromide have been wholly unsuccessful (147, 150). Selenium dehydrogenation of solanidine and solanocapsidine gave pyridine (150), 2-ethyl-5-methylpyridine (151), 2-methyl-5-ethylpyridine and 4-methyl-2-ethylpyridine (138) beside CXXVIII. For solanidine these results have been formulated as follows:
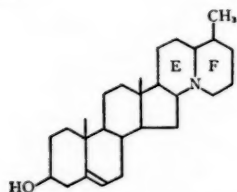
CXLVIII. Soltys (152)
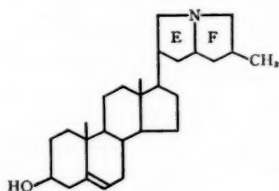
CXLIX. Clemo (153)
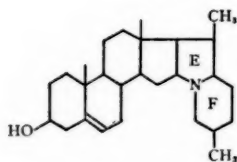
CL. Prelog (151)
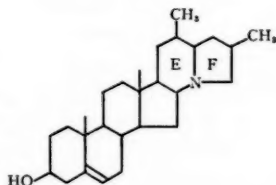
CLI. Rochelmeyer (154)

Formula CXLIX is not likely because no *trans*-androsterone acetate has been obtained from dihydrosolanidine acetate on oxidation with chromic acid (154). On the other hand such pyrrolizidine ring systems were recently found in the alkaloids of *Senecio, Heliotropium,* and similar species (155, 156). In formulae CXLIX and CL the sequence of carbon atoms in rings E and F is the same as in cholesterol. For-

mula CL would furnish the simplest explanation for the formation of 2-ethyl-5-methylpyridine in the selenium dehydrogenation (151).

Solasodine contains two alcoholic hydroxyl groups; according to Briggs et al. (134), the second hydroxyl group is located in the basic part of the molecule. Assuming formulation CXLIX as the correct expression for the carbon skeleton of these alkaloids, solasodine is formulated as a quaternary hydroxide (CLII) which can also react as a carbinol-amine (CLIII):
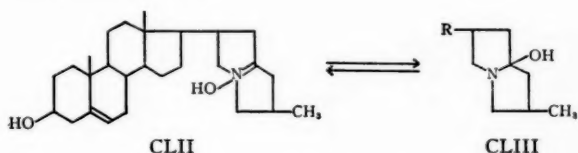
The quaternary formulation explains the alkaline reaction to litmus in alcoholic solution and also the formation of solasodine hydroiodide when treated with methyl or ethyl iodide (134). The normal salts, with the exception of the nitrite are derived from formula (CLIII). If solasodine is hydrogenated with palladium or small amounts of platinum the double bond in position 5,6 is saturated. With more platinum two moles of hydrogen are absorbed. The resulting tetrahydrosolasodine (CLIV) (134, 141) still retains two oxygen atoms and yields a diacetate (141). Briggs et al. (134) formulate the hydrogenation as follows:
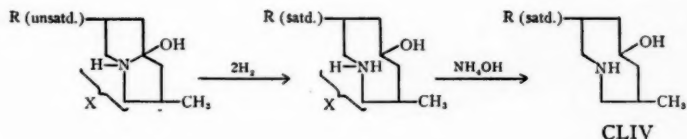
In case a quaternary hydroxide (CLII) had been hydrogenated a dihydrosolasodine with only one oxygen atom might have been expected (134). When solasodene [corresponding to (CXXXV), see above] was hydrogenated, three moles of hydrogen were absorbed without elimination of oxygen. It is also suggested that here hydrogenation involves fission of the heterocyclic rings.

Nothing is known of the basic part of solauricidine and no further experimental work has been done on the constitution of solanocapsine,

the structure of which is purely speculative. Instead of Barger's formula (CLV) (138) Rochelmeyer (141) proposes structure (CLVI).

CLV   CLVI
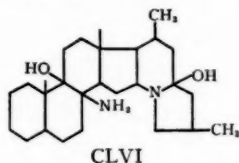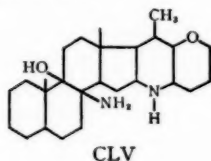
Other alkaloids with a steroid structure.—It is doubtful whether the alkaloids of the Chinese drug "pei-mu," identified as *Fritillaria Roylei* [cf. (3)] are sterol derivatives (157 to 160). But most probably the alkaloids (or some alkaloids) of *Veratrum* [cf. (3)] belong to the steroid group. Recently Jacobs & Craig (161, 162) isolated cyclopentenophenanthrene (and not the γ-methyl homologue hitherto obtained from all other steroids) from isorubijervine after selenium dehydrogenation. 2-Ethyl-5-methylpyridine was obtained previously as a typical cleavage product from cevine (163), jervine (164), and protoveratrine (165).

Addendum.—Prelog's formula of solanidine (CL) has meanwhile been proved by partial synthesis of allosolanidane-3(β)-ol (CXLI) (147) from sarsasapogenin. This remarkable conversion already announced by Prelog & Szpilfogel (147) was carried out by Uhle & Jacobs (166) in the following manner. The dioxime of sarsasapogenoic acid (CLVIII) obtainable from sarsasapogenin (CLVII)

CLVII   CLVIII   CLIX

CLX   CXLI
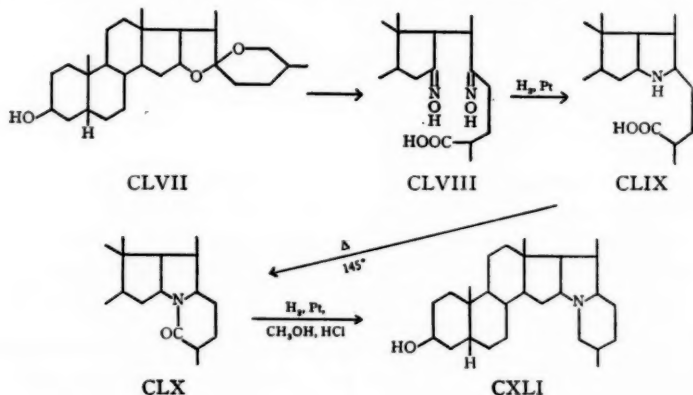
yielded the secondary amino acid (CLIX) on hydrogenation. The latter lactamized readily on heating to give the neutral lactam (CLX) which was easily reduced with an active platinum oxide catalyst in dilute hydrochloric acid and methanol to the tertiary base (CXLI).

Since the veratrine alkaloids give the same degradation product, 2-ethyl-5-methylpyridine, and, in general, are structurally analogous to solanidine, the formulation of the heterocyclic portion of the molecule in the tertiary bases of the veratrine series as an octahydropyrrocoline may be considered quite certain (166).

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CARBOHYDRATE METABOLISM

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A complete survey of papers published in 1945 was made difficult by the inaccessibility of many European journals. With few exceptions, a review of the carbohydrate metabolism of microorganisms could not be included because of the limitation of space.

The increasing importance of isotopes as a tool in biochemistry is reflected in the rapid advances which have been made in intermediary carbohydrate metabolism with this technique. Isotopes have also been used to great advantage in demonstrating reversibility of enzymatic reactions, particularly those involving carbon dioxide fixation or phosphate transfer. The common metabolic pool for carbon fragments derived from the three principal foodstuffs and their common oxidative pathway has made it impossible to discuss the intermediary metabolism of carbohydrates without some reference to that of the other foodstuffs.

The gap which often exists between results obtained in experiments on the whole animal and on isolated enzyme systems is being bridged to some extent. Here again the use of isotopes plays a leading role, but a unified picture will not emerge until the mechanism of the regulatory effect of hormones on enzyme systems is more clearly understood.

The usual abbreviations have been used: ATP and ADP for adenosinetriphosphate and adenosinediphosphate; TPN and DPN for tri- and diphosphopyridine nucleotide; and P for phosphate.

TRACERS IN THE STUDY OF INTERMEDIARY METABOLISM AND CARBON DIOXIDE FIXATION

Until recently one of the reactions in the glycolytic scheme has been regarded as irreversible, namely, phosphopyruvate $+$ ADP $\rightarrow$ pyruvate $+$ ATP. It was assumed that this irreversible step was circumvented by a conversion of pyruvate to a C_4-dicarboxylic acid and conversion of the latter to phosphopyruvate, a sequence of reactions which would account for the incorporation of isotopic carbon (from bicarbonate) in glycogen. From available thermodynamic data

an exchange of phosphate between ATP and phosphopyruvate seemed possible. Previous negative results were due to the fact that potassium ions were not added, K^+ being necessary for the transfer of phosphate in the above reaction. Lardy & Ziegler (1) have now demonstrated, by measuring the distribution of P^{32} and by chemical measurements, that pyruvate and ATP can form phosphopyruvate in the presence of K^+, making unnecessary the assumption that the formation of a 4-carbon compound is an obligatory intermediate.

The study of individual reactions of the tricarboxylic acid cycle (which is concerned with the oxidation of carbohydrate and fat) has been continued by Ochoa (2, 3). Using heart muscle extract, he has found that the oxidation of isocitric to α-ketoglutaric acid involves two enzymatic reactions, both of which are reversible. The first reaction, catalyzed by isocitric dehydrogenase, yields oxalosuccinate in the presence of TPN. The second reaction, oxalosuccinate $\rightleftarrows$ α-ketoglutarate $+ CO_2$, requires Mn^{++} and its discovery represents an important advance in our knowledge of the biological utilization of carbon dioxide. The isocitric dehydrogenase system is linked to the cytochrome system through cytochrome-c reductase which catalyzes the reoxidation of reduced TPN by cytochrome-c (4).

In previous work on carbon dioxide fixation in animal tissues the assumption was made that pyruvate reacts with carbon dioxide to form oxaloacetate. The fixation of $C^{13}O_2$ by pigeon liver extract, with pyruvate and fumarate as substrates, has been investigated by Wood et al. (5). The isotopic carbon was found in the carboxyl groups of fumarate, malate, pyruvate, and lactate in about equal concentrations, but an attempt to demonstrate an exchange between $C^{13}O_2$ and oxaloacetate gave a negative result. This left open the question of the primary product of the fixation reaction, although the results did not exclude the reaction previously proposed, namely, pyruvate $+ CO_2 \rightleftarrows$ oxaloacetate. Direct proof for this reaction in animal tissues (it had been demonstrated before in bacteria) has now been obtained by Utter & Wood (6). They found C^{13} in the carboxyl group of oxaloacetate adjacent to the methylene group when pigeon liver extract was incubated anaerobically with pyruvate, oxaloacetate, Mn^{++}, $NaHC^{13}O_3$, and ATP. Addition of ATP increased the fixation of carbon dioxide in dialyzed extracts, but it is not yet clear how it participates in the reaction.

Two coenzyme-linked reactions which lead to the formation of oxaloacetate or oxalosuccinate (the intermediates which are in equi-

librium with carbon dioxide) have been studied by Moulder *et al.* (7). Malate was oxidized to oxaloacetate while pyruvate was reduced to lactate. In pigeon liver extract either DPN or TPN could serve as coenzyme, and this was also true for the pairs, isocitrate + pyruvate → oxalosuccinate + lactate. As anticipated by the above authors, and as proved by Ochoa (3), oxalosuccinate is decarboxylated by a separate enzyme which does not act on oxaloacetate.

The reductive carboxylation in certain bacteria, acetylphosphate + CO_2 + H_2 ⇌ pyruvate + phosphate, has been investigated by Lipmann & Tuttle (8) and Utter *et al.* (9) with P^{32} and C^{13}; a rapid exchange of P^{32} between free and bound phosphate and of C^{13} between carbon dioxide and the carboxyl group of pyruvate has been observed. Lipmann points out that a reaction of this type may be a key reaction in photosynthesis in plants and he proposes a scheme, based on known enzymatic reactions, which satisfies the energetic requirements for the reduction of carbon dioxide to the carbohydrate level.

The deposition of glycogen in the liver is accompanied by the fixation of carbon dioxide. The question of the position of the fixed carbon in the glucose residue in glycogen has been investigated by Wood *et al.* (10, 11). They administered glucose and $C^{13}O_2$ (as bicarbonate) to fasting rats and isolated liver glycogen which was then hydrolyzed to glucose. Bacterial and chemical degradation yielded the important information that all of the isotopic carbon was in positions 3 and 4. In order to explain fixation in these two positions it is necessary to assume that part of the glucose residues in glycogen is formed from C_3 + C_1 or C_2 + C_1 addition products. The combination of pyruvate and carbon dioxide to oxaloacetate would lead to the formation of phosphopyruvate and to the labeling of positions 3 and 4 by a reversal of the reactions of the glycolytic cycle. A reductive C_2 + C_1 addition involving acetylphosphate has so far been observed only in certain bacteria (see above). Although Lipmann & Tuttle (12) have now succeeded in demonstrating the formation of what appears to be acetyl phosphate (from acetate and ATP) in pigeon liver extract, there is no evidence for the occurrence of C_2 + C_1 addition in animal tissues. Metabolic pathways of C_2 compounds will be discussed below, because they have a considerable bearing on the problems under discussion.

The search for the reactive C_2 intermediate which is formed during oxidation of pyruvate as well as of fatty acids continues. The further

fate of the 2-carbon fragments in liver was found to depend on the availability of dicarboxylic acids; in their absence the fragments condensed to form acetoacetate, in their presence the 2-carbon fragments reacted with oxaloacetate and thus entered the tricarboxylic acid cycle (13). Acetate itself which may be a stabilization product of the reactive C_2 intermediate entered the tricarboxylic acid cycle, as shown by labeling experiments (14, 15, 16).

The postulated common pathway for the oxidation of carbohydrate and fat has a number of important consequences. As pointed out by Buchanan et al. (14), the oxidation of a C_2 residue (derived from pyruvate, acetoacetate, acetate, or from naturally occurring fatty acids) through the tricarboxylic acid cycle does not result in a net gain of carbohydrate, i.e., $C_2 + C_4 \rightarrow 2CO_2 + 2H_2O + C_4$ leaves the concentration of oxaloacetate (which can be converted to carbohydrate via phosphopyruvate) unchanged. A net gain in carbohydrate would result if acetate were convertible to pyruvate (by reductive $C_2 + C_1$ addition), since the latter can combine with carbon dioxide to form oxaloacetate or can be converted directly to phosphopyruvate (1).

A consideration of the oxidation of isocitrate (derived from a $C_2{}^{13} + C_4$ condensation) to succinate,

$$C^{13}OOH \cdot C^{13}H_2 \cdot CH \cdot CHOH \cdot COOH \rightarrow$$

$$COOH$$
$$C^{13}OOH \cdot C^{13}H_2 \cdot CH_2 \cdot COOH + 2CO_2,$$
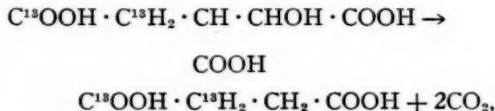
shows that the carbon dioxide formed when one C_2 residue is oxidized does not arise from the C_2 residue itself. The fact that succinate can be converted to carbohydrate would explain why isotopic acetate carbon has been found to be incorporated in liver glycogen. Since succinate is a symmetrical molecule, all positions in glucose derived from glycogen would be expected to be labeled. This has actually been demonstrated by Lorber et al. (15, 16), by feeding glucose and acetate labeled in both carbons to rats. With carboxyl-labeled acetate they found the isotopic carbon only in positions 3 and 4 of the glucose molecule.

The incorporation of fatty acid carbon in glycogen and in amino acids (17) represents a redistribution of carbon as it passes through a common metabolic pool and in this sense all three foodstuffs are interconvertible. From the standpoint of the economy of the organism

and for the interpretation of certain metabolic disturbances such as diabetes it is important to emphasize that the isotope studies do not support the view that extra carbohydrate is formed during the metabolism of fatty acids (14).

Stetten & Klein (18, 19) have continued work on the uptake of deuterium into glycogen and fatty acids from deuterium oxide administered to the animals. Liver glycogen isolated from previously fasted rats contained 25 per cent of the deuterium content of the body water, while three hours after feeding of glucose or lactate or injection of epinephrine, the respective figures were 38, 57, and 56 per cent, as compared to an estimated maximum of 66 per cent if all the stable, carbon-bound hydrogens were exchanged. The much lower isotope content after glucose than lactate feeding indicates that a considerable part of the glucose is converted directly to glycogen without passing through a C_3 stage. The fact that the liver glycogen after epinephrine injection had the same high isotope content as after lactate feeding supports the hypothesis of the lactic acid cycle, according to which blood lactate derived from muscle glycogen is converted to liver glycogen.

The galactose excreted in the urine of rats fed this sugar in the diet does not contain deuterium, indicating that the carbon-bound hydrogen of hexoses does not exchange with deuterium oxide in the body fluids (20). Deuterium found in urinary glucose is therefore a measure of glucose synthesis from smaller fragments, that is, of gluconeogenesis. In the alloxan-diabetic rat on a carbohydrate diet, about 75 per cent of the urinary glucose was of dietary origin and only 25 per cent represented gluconeogenesis. In a phlorhizinized rat the respective figures were 65 and 35 per cent. These figures do not favor the overproduction theory of pancreatic diabetes. The glycogen synthesized in muscles after administration of glucose plus insulin was low in deuterium and was therefore largely formed from glucose directly. This supports the idea that the process favored by insulin is the utilization of glucose and conversely that there exists in diabetes an under-utilization of glucose (18, 19).

A striking difference was noted in the amount of fatty acids synthesized on a carbohydrate diet; namely 1.9 gm. per day in normal, 0.1 gm. in alloxan-diabetic, and 1.0 gm. in phlorhizinized animals (18, 19). An alloxan-diabetic rat fed an alanine diet also failed to synthesize fatty acids and its sugar excretion in the urine was very low (0.3 gm. as compared to 14.2 gm. on a carbohydrate diet); this

is of considerable importance for the interpretation of this experiment, since the rate-determining step is probably the deamination of alanine to pyruvate which can then serve as the source of urinary glucose as well as of fatty acids.

Sacks (21), using P^{32} as tracer, has measured the effect of injection of glucose and glucose + insulin on the phosphate turnover in cat muscle during rest and after recovery from exercise. He reports an increase in the rate of turnover in phosphocreatine, ATP, and hexose-6-phosphate in resting muscle under the influence of insulin. Kaplan *et al.* (22) have demonstrated aerobic phosphorylation in kidney slices by means of P^{32}.

ENZYMATIC MECHANISMS AND TISSUE METABOLISM

Many of the enzymes concerned with carbohydrate metabolism are known to be inhibited by reagents which react with sulfhydryl groups and to be activated by reducing agents such as cysteine and glutathione. Some of these enzymes have been investigated by Barron & Singer (23) with a variety of reagents showing different reactivity toward sulfhydryl groups, which provides a means of classification.

One of these enzymes, glyceraldehyde phosphate dehydrogenase, which according to these criteria would be classified as an "SH enzyme," has been obtained in crystalline form from rabbit muscle (24). The method of preparation is very simple and the yield is high, about 200 mg. per 100 gm. muscle. This crystalline enzyme shows very little activity unless a reducing agent is added. The equilibrium of the reaction, $HC = O + P + DPN \rightleftarrows P - O - C = O$ + DPNH, has been investigated by Drabkin & Meyerhof (25) with yeast glyceraldehyde phosphate dehydrogenase, by spectrophotometric methods. The mechanism of phosphorylation in the above reaction had not been clarified. Evidence has now been obtained which favors the assumption that an unstable addition product is formed between glyceraldehyde phosphate and inorganic phosphate prior to the formation, by oxidation, of glyceric acid diphosphate.

Colowick & Price (26 to 29) have investigated the hexokinase and the analogous phosphofructokinase reaction in rat muscle extract. In addition to Mg^{++}, two other components, guanine and dihydrocozymase, were found to be necessary for the activity of these enzymes. The former could be removed by dialysis, the latter by aging of the extracts at pH 6. Although dialyzed extracts could be reactivated by inorganic

phosphate, there was a lag period before activity set in. It was shown that guanine is formed during the lag period from ribonucleic acid present in the dialyzed extract by an enzymatic reaction described in another section. Inorganic phosphate was necessary for this reaction, and since iodoacetate inhibited it, phosphate did not activate a dialyzed, iodoacetate poisoned extract, while guanine did. In aged and dialyzed extracts, either component alone was without effect, while addition of both guanine and dihydrocozymase restored activity.

Yeast hexokinase has been prepared in crystalline form (30, 31). Gottschalk (32) has presented some evidence that only the furanose form of fructose (free hydroxyl group in the 6 position) reacts with yeast hexokinase; in this case fructose-6-phosphate would be the primary product formed. It is of interest in this connection that the formation of fructose-1-phosphate has been observed in liver (33, 34) and intestinal mucosa (34) during absorption of fructose; this suggests a reaction with the pyranose form of fructose.

Spiegelman & Nozawa (35) have investigated the endogenous respiration of several strains of yeast. The respiratory quotient was close to unity and there was no carbon dioxide production anaerobically, but when glucose was added fermentation occurred both aerobically and anaerobically. From this it is concluded that the carbohydrate reserves present in the intact yeast cell are accessible to oxidation but not to fermentation. Lindegren (36) investigated the conditions which lead to the deposition of glycogen in yeast cells and induce a state of dormancy. Spiegelman (37) has presented detailed experiments on the adaptation to the fermentation of galactose and melibiose by yeast and has reached important conclusions with respect to the mechanism of genetic control of adaptation.

Meyerhof (38) has shown that the two phases of glucose fermentation in yeast extract, the fast initial period and the slow second period, can be explained by the absence of adenylpyrophosphatase. The latter enzyme is essential in the second period, when all the glucose has been used up, since it provides the necessary phosphate acceptor by splitting ATP to ADP and thereby determines the rate of fermentation of previously accumulated hexosediphosphate. This was proved by adding potato adenylpyrophosphatase during the second period; fermentation increased rapidly and attained the rate of the first period.

Destruction of coenzymes is another factor which has to be kept in mind in the study of fermentation reactions. This is clearly shown

in the work of Utter *et al.* (39, 40) on glycolysis in brain extract. Addition of nicotinamide (which protects DPN from destruction by nucleotidase) gave sustained fermentation rates which were proportional to the amount of extract used. With this improved system (for the details of which the original should be consulted), the authors (41) were unable to confirm the claim that the glycolytic activity of the brain of mice infected with poliomyelitis virus was lower than that of normal brain. Glycolysis in nervous tissue was shown to proceed over the same intermediate steps as in muscle.

On the basis of a study by Mann (42), spermatozoa can be added to the list of cells for which a phosphorylating type of glycolysis has been found; it is of importance that in this case, owing to the ease of penetration of added substances, all the essential steps of glycolysis could be demonstrated on intact, motile cells. A close correlation was found between motility and glycolytic activity. The effect of various carbohydrate derivatives on respiratory activity and motility of mammalian spermatozoa has been investigated by Lardy *et al.* (43). The authors assume that under certain conditions, oxidation of phospholipids stored in the spermatozoa can serve as the source of energy for phosphorylation.

Potter (44) found that with succinate as oxidizable substrate, rapid aerobic phosphorylation of creatine with dilute kidney homogenates required addition to the reaction mixture of cytochrome–c, ATP, and Mg^{++}. Phosphate uptake occurred over a wide range of concentration of succinate (2 to 33 mM) whereas malate or oxaloacetate was effective only within a restricted range of concentration (about 3 mM). With heart and skeletal muscle homogenates, phosphate uptake was more rapid with oxaloacetate than with succinate (45).

Perfusion of cow mammary gland with high concentrations of blood sugar led to considerable deposition of glycogen. When unperfused glands were incubated no lactose was formed while incubation of perfused glands led to disappearance of glycogen and formation of lactose (46).

Oxygen consumption was found to be inversely related to the glycogen content of liver slices, probably because of dilution of active tissue with glycogen and water (47). Crandall (48) studied the relationship between oxygen uptake, R.Q., and glycogen formation from glucose in rabbit liver slices suspended in Ringer's solution, pH 8.5. A positive correlation was found between the rate of glycogen formation and the height of oxygen consumption and R.Q. From

this the author concludes that at a low R.Q. when fatty acids are oxidized to ketone bodies, fewer high energy phosphate bonds are generated than at a high R.Q., when carbohydrate is oxidized.

Phosphorylation of glucose and fructose in liver extracts of different species has been investigated (49). The claim that cat liver cannot phosphorylate glucose may be due to factors which are difficult to control in extracts or in perfusion experiments rather than to an essential species difference. Fantl & Rome (50) have presented some evidence for the existence of a specific glucose-6-phosphate phosphatase in liver, an enzyme directly concerned with blood sugar formation. This is based on a comparison of the pH optima for dephosphorylation of different substrates. Jeener (51) observed a lower glycogenolytic rate in rat liver slices under anaerobic than aerobic conditions and found that this correlated with the adenylic acid content of the slices and hence with phosphorylase activity which is influenced by the adenylic acid concentration.

Phosphorylase-b, obtained by the action of the PR enzyme on crystalline phosphorylase-a, has been crystallized in the form of rhomboid plates. The action of the PR enzyme on phosphorylase-a was found to consist in the splitting off of a prosthetic group which contains about 0.7γ of organic phosphate per mg. protein. The fact that phosphorylase-b requires the addition of adenylic acid for activity, while phosphorylase-a is active without it made possible the determination of the two enzymes in mixtures. Resting muscle was found to contain mainly phosphorylase-a. During strong muscular contraction most of the phosphorylase-a was converted to b by the action of the PR enzyme *in vivo*, a change which was shown to be reversible during recovery from stimulation. The mechanism represented by the action of the PR enzyme is found with other enzymes and may have a regulatory function; in the above case it may be connected with the preservation of the glycogen stores of muscle in fatigue (52).

ENZYMATIC SYNTHESIS OF GLYCOSIDIC BONDS

Schoch (53) has summarized methods for the separation of starch into two components, a linear and a branched fraction, and has described their properties. Further subfractionation of these two components, amylose and amylopectin, was described by Kerr (54). Polysaccharides having the general properties of these two components can be synthesized enzymatically. Only one enzyme, phosphorylase, is needed for the formation of a linear polysaccharide, while two enzymes

are involved in the formation of a branched polysaccharide. A branching factor which acts together with phosphorylase was first demonstrated by Cori & Cori (55) in extracts of liver and heart and has now also been found by Haworth *et al.* (56, 57) in potato extract; the latter authors do not acknowledge previous work in this field.

A symposium has appeared (58) containing articles on molecular constitution and on mechanisms of enzymatic synthesis of polysaccharides, disaccharides, and nucleosides. New data are presented on the differentiation of polysaccharides by means of muscle and potato phosphorylase. This is based on the different activating power of various native polysaccharides and their hydrolysis products for the two enzymes. The potato enzyme could be activated by achroodextrins, e.g., those obtained on acid hydrolysis of the cyclic Schardinger dextrins or of the amylose fraction of starch. In both cases the average chain length of the dextrins was found to be five glucose units, when the enzyme activity reached its maximum. The hydrolysis products of Schardinger dextrins or of amylose did not activate the muscle enzyme. The latter requires a large molecule with many branches (glycogen or amylopectin) for activation. Acid hydrolysis quickly destroyed the activating power of glycogen or amylopectin for muscle but not for potato phosphorylase. With the potato enzyme there occurred a four-fold increase in activating power on short acid hydrolysis of glycogen, while acid hydrolysis of starch gave only a slight increase (59).

Further support has been advanced for the thesis that polysaccharide synthesis consists in a lengthening of existing polysaccharide chains. That the length of the newly formed chains increases progressively with time was shown by simultaneous determination of iodine coloration and chain length during enzymatic synthesis. The chain length of the synthetic polysaccharide was calculated from the molar ratio, 1-ester used in enzymatic reaction/terminal glucose units of activator, and found to agree with that calculated from end group determinations (59). Sumner *et al.* (60), Hidy & Day (61), and Kusin & Ivanov (62) have reported experiments in which the chain length of the newly formed polysaccharide, as judged from the iodine color, was dependent on the amount of activator added. Short chains were formed when a large amount of activator was added and vice versa, indicating that the enzyme was building onto the added polysaccharide chains.

Kalckar (63, 64) has discovered a nucleoside phosphorylase in liver

which forms ribose-1-phosphate and hypoxanthine or guanine from the respective nucleosides and inorganic phosphate. The reaction was found to be reversible and should prove to be of great importance in the field of enzymatic synthesis of nucleotides and nucleic acids. Colowick & Price (29) have found another phosphorylase in muscle which splits off guanine and introduces phosphate in place of guanine in ribonucleic acid. As in the reaction of Kalckar, ribose-1-phosphate is formed, except that here it is linked to the rest of the nucleic acid molecule. This reaction was also found to be reversible.

Doudoroff's bacterial phosphorylase which forms sucrose from glucose-1-phosphate and fructose is another example of this class of enzymes. In all these cases a 1-phospho sugar reacts with another molecule to form a glycosidic bond and inorganic phosphate or, in the reverse direction, a glycosidic bond is split by reacting with inorganic phosphate. Doudoroff (65) has been unable to demonstrate a sucrose phosphorylase in higher plants and he discusses several other possible mechanisms for the formation of sucrose.

The bacterial levulan synthesis (66) involves the exchange of one glycosidic bond for another, fructose-2-glucose (sucrose) being analogous to glucose-1-phosphate in the phosphorylating mechanisms of polysaccharide synthesis. Doudoroff (67) has shown that the levulan synthesis is reversible and in this case glucose takes the place of inorganic phosphate in the splitting of the glycosidic bonds of the polysaccharide chain. Another reaction of this type is the bacterial dextran synthesis in which sucrose (glucose-1-fructose) reacts as a glucoside in the building up of a polysaccharide chain, while fructose is set free. Although soluble, these polysaccharides may be comparable to the capsule polysaccharides of other bacterial forms. It is of interest in this connection that Aschner *et al.* (68) found that certain capsulated yeasts contain in their capsules a polysaccharide which gives a strong blue color with iodine; the substance was also found in the cell-free medium and was identified as amylose.

ALLOXAN DIABETES

Several methods have been described for the determination of alloxan (69 to 72). Its rapid destruction at the pH and temperature of the blood (half-life about four minutes) and its reactivity with thiol and other groups explains the almost complete disappearance of alloxan from the blood five minutes after intravenous injection (69,

72). According to several investigators the main decomposition products of alloxan in blood, alloxanic and dialuric acids, are not diabetogenic. It was therefore to be expected that damage to the islet tissue would occur in the first few minutes after injection. This was proved by Gomori & Goldner (73) in dogs. Clamping a portion of the pancreas during and for one to six minutes after injection protected that portion from histological changes, while typical degenerative changes of β-cells were observed in the unclamped portion.

Protection from histological changes was also afforded by injecting just prior to alloxan certain substances which are known to combine with it, such as 3,4-diaminotoluene, orthophenylenediamine, and sodium bisulfite (74). Brückmann & Wertheimer (75) have tested the diabetogenic effect of various alloxan derivatives by injecting them intravenously in rats. They report also that alloxan forms methemoglobin *in vitro*.

The mechanism of the initial transitory hyperglycemia after injection of alloxan is still under discussion. Houssay *et al.* (76, 77) report that an initial hyperglycemia was not observed in hepatectomized dogs, but that it did occur in adrenalectomized dogs, hence they assume a direct action of alloxan on the liver. In rabbits (78) and rats (79) adrenalectomy prevented the initial hyperglycemia. Kirschbaum *et al.* (79) attribute this effect to the removal of the adrenal cortex rather than the medulla, and they also found that hypophysectomy prevented the initial hyperglycemia. Disagreement also exists with respect to the mechanism of the second or hypoglycemic phase which is often severe enough, especially in rabbits, to cause convulsions. Houssay *et al.* (76, 77) injected alloxan in dogs depancreatized one half hour previously and observed hypoglycemia one to three hours after injection; they conclude that the hypoglycemia was due to an extrapancreatic effect of alloxan. However, this effect did not occur in dogs depancreatized twenty-four to forty-eight hours previously. In partially depancreatized rabbits severe hypoglycemia did not develop when alloxan was injected three hours after operation, while the controls died in hypoglycemic convulsions (80). Shipley *et al.* (81) report a marked delay in the hypoglycemic response when alloxan was injected in vagotomized, sympathectomized dogs.

There is general agreement that the permanent hyperglycemia results from loss of insulin production by the pancreas. Houssay *et al.* (76, 77) found that the pancreas from dogs injected with alloxan twenty-four to forty-eight hours previously, when grafted in the neck

of depancreatized dogs by vascular anastomosis, failed to secrete insulin in four cases, showed very reduced secretion in three cases, and gave normal secretion only in one case. Direct assay of the pancreas by others has also shown a greatly reduced insulin content.

Histological examinations in various species have shown that the β-cells disappear, while the α-cells are not affected (78, 82, 83). Some differences have been observed between alloxan diabetic dogs and depancreatized dogs. Houssay et al. (76, 77) note that the final rise in blood sugar was sometimes higher in the former than in the latter animals. Thorogood & Zimmermann (84) also found that glycosuria was more severe and the insulin requirement higher in alloxan diabetes than after pancreatectomy. Nevertheless, the alloxan diabetic dogs survived longer without insulin and they showed low ketonuria and did not develop coma. Removal of the pancreas from alloxan diabetic dogs reduced glycosuria and the insulin requirement. When insulin was withheld from such animals, ketosis and coma developed rapidly. The suggestion is made that these differences may be due to the elaboration of a separate hormone by the α-cells of the pancreas which increases blood sugar and prevents ketosis.

The diabetogenic dose varies in different mammals (78), the dog being the most sensitive and the rabbit and rat the most resistant species. There is also a considerable individual variability and the margin between the diabetogenic and fatal dose is small. In rats following intraperitoneal injection of 200 mg. per kg., about 60 per cent developed moderate to severe diabetes, while 40 per cent either died in a few days or failed to become diabetic. That the functional state of the β-cells may play a role in their susceptibility to the toxic effects of alloxan, is indicated by an observation of Kass & Waisbren (85). They report that when rats were first starved for forty to sixty hours and were then given 175 mg. per kg. subcutaneously, the mortality was low and the animals developed chronic hyperglycemia in 90 to 95 per cent of the cases. Glucose given six hours before alloxan diminished the susceptibility of the starved rats. A group of nonfasting rats previously found refractory to alloxan was made susceptible when the animals were fasted for 60 hours prior to the injection.

Although alloxan was shown to pass through the placenta of the rat, it did not induce diabetes in the embryos when the injections were given four days to six hours before parturition (86). Alloxan diabetes induced in twenty-six day old rats stunted their growth (87). Repeated small doses of alloxan injected over four weeks caused no

hyperglycemia in rats but resulted in a lowered tolerance to glucose
which was permanent. Injections of anterior pituitary extract caused
hyperglycemia in these animals (88). In rats dying from alloxan, in
what was probably diabetic coma, there was an increase in plasma and
liver inorganic phosphate and a decrease in the total acid-soluble phos-
phate of the liver. The latter was due to loss of ATP as well as of
other organic phosphates. Insulin in very large doses reversed some
of these changes and caused a marked increase in liver glycogen and
an improvement in the clinical condition of the animals (89).

Degenerative changes have been described in the basophile cells of
the pituitary and in the adrenal cortex following large doses of al-
loxan (90). Adrenal cortical lesions have also been described in rab-
bits (91). Adrenalectomy in rats with alloxan diabetes reduced hyper-
glycemia and glycosuria (92).

According to Scott *et al.* (93), birds with the exception of pigeons
did not develop hyperglycemia and the β-cells of the pancreas in ducks
and other species did not show lesions or only minimal changes, while
Mirsky (94) reports irregular necrotic changes in the islet tissue of
ducks. In pigeons the blood sugar rose to 300 to 400 mg. per cent
within twenty-four to forty-eight hours following the injection of 150
to 200 mg. per kg. intravenously, a dose which was usually fatal. One
half this dose usually caused no hyperglycemia. An additional effect
in pigeons was the occurrence of visceral gout with sodium urate
deposits on all serous membranes (95).

Several summarizing articles have been published (96, 97).

ENDOCRINE GLANDS

Adrenals.—Doetsch (98) has measured the disappearance of in-
organic phosphate in fluoride-poisoned muscle and liver brei of nor-
mal and adrenalectomized rats, finding a smaller disappearance in
the latter. This is regarded as being indicative of a decrease in glyco-
gen phosphorylation in the tissues of adrenalectomized rats. Accord-
ing to Montigel (99) the decreased glycogen phosphorylation in mus-
cle brei of adrenalectomized rats was brought back to normal by addi-
tion of desoxycorticosterone. Helve (100), using a similar technique,
could find no difference in phosphorylation between muscles of normal
and adrenalectomized rats.

Ingle *et al.* (101, 102) found that the daily injection of 5 mg. of
17-hydroxycorticosterone or 17-hydroxy-11-dehydrocorticosterone in

rats on a high carbohydrate diet caused glycosuria. After cessation of the injections the animals again became normal. The glycosuria induced by steroid injection was highly resistant to insulin. Glycosuria could also be produced by daily administration of 7 mg. of an adrenocorticotropic hormone preparation (103).

Vogt (104) reports that epinephrine given in physiological doses by intravenous infusion in dogs caused an immediate and long lasting output of adrenal cortex hormones, as shown by the effect of injection of adrenal venous blood on the survival of adrenalectomized rats exposed to cold. This emphasizes again the fact that certain changes observed in carbohydrate metabolism after the injection of one hormone may in reality be due to the effect of another hormone. In view of the effects of cortical steroids reported above, it seems possible that the decreased blood sugar utilization observed after epinephrine injection is due to increased steroid secretion. That epinephrine induces changes in the adrenal cortex is also emphasized by the finding of Long & Fry (105) that there occurred a decrease in the cholesterol and ascorbic acid content, an effect which was not observed in hypophysectomized rats. Abelin (106) has found a decrease in adrenal cholesterol six to eight hours after a carbohydrate meal.

Olson *et al.* (107) have investigated the glycogenic activity of six crystalline hormones of the adrenal cortex. 17-Hydroxycorticosterone was the most active in causing deposition of liver glycogen in fasted, adrenalectomized rats, while desoxycorticosterone acetate was inactive. Simultaneous administration of corticosterone and 17-hydroxycorticosterone resulted in an augmentation of potency.

Pancreas.—Pancreatectomy in two calves produced only a mild form of diabetes, similar to that in the goat and in the hypophysectomized dog. Hyperglycemia and glycosuria depended on food intake. During fasting the blood sugar levels were normal and there was no ketonuria (108). This low endogenous gluconeogenesis is probably related to anterior pituitary function which may vary in intensity in different species. A case of total pancreatectomy (because of carcinoma) has been reported in man. The patient survived fourteen weeks and showed fasting dextrose: nitrogen ratios of 2.4 to 3.6 (109). In a cat with spontaneous severe diabetes definite histological changes were observed in the islet tissue (110).

Gaebler & Ciszewski (111) found that the insulin dose which was adequate for the control of glycosuria in depancreatized dogs on a purified diet with a yeast supplement, became inadequate when yeast

was omitted. Vitamins of the B-group could be substituted for yeast. Partially depancreatized rats, when given several choices, selected a diet rich in fat and protein and avoided carbohydrate; they had less polyuria, lower blood sugar, and lost less weight than on a diet rich in carbohydrate (112).

Root (113) has surveyed the treatment of some 600 cases of diabetic coma and advises strongly against the early use of glucose. Mattar *et al.* (114) deny the possibility that the aggravation of diabetes by infection is due to gluconeogenesis at the site of infection.

Pituitary.—Bennet & Perkins (115) have shown that the pituitary factor which is necessary for the maintenance of muscle glycogen during fasting can act in the absence of the adrenals, since its injection was as effective in hypophysectomized, adrenalectomized as in hypophysectomized rats.

Conn & Louis (116) prepared a crude pituitary extract which when injected into dogs was diabetogenic after a latent period of several weeks. Amounts equivalent to 24 to 40 gm. of pituitary were injected daily. The same extract (40 gm.) was injected into two patients who had pancreatic islet adenomas and low blood sugar levels. The extract caused a further lowering of the blood sugar level. When the injections were discontinued after about twenty days, the blood sugar rose to the pre-injection level or higher. The glucose tolerance was not affected by the injections, while in the dogs it was reduced during the latent period. The authors conclude that their extract contained an insulotropic principle, the activity of which was brought to light in the patients because of the presence of abnormally large amounts of islet tissue. This same persistent stimulus is believed to lead eventually to a decrease in insulin production and diabetes in dogs. Ogilvie (117) injected rabbits for about two weeks with large amounts of anterior pituitary extract. The islets in these animals averaged more than twice the weight of those of controls. The animals gained in weight despite a reduced caloric intake. Some developed temporary glycosuria and ketonuria.

Young (118) injected puppies for four months with a diabetogenic anterior pituitary extract; their growth was accelerated during the latent period. Later three out of four puppies which had become diabetic were given in addition to the pituitary injections large doses of insulin, whereupon they resumed growth. Diabetic dogs in which pituitary injections were continued required as much as 80 units of insulin per kg. for control of the diabetes.

Keller *et al.* (119) have carried out a study, controlled by careful histological examinations, of the effects of varying degrees of hypophysectomy in the dog on insulin sensitivity, adrenal function, and severity of diabetes after pancreatectomy (Houssay effect). Removing the anterior lobe completely, but leaving the stalk intact decreased insulin tolerance, but did not cause adrenal atrophy or an amelioration of diabetes upon subsequent pancreatectomy. Total removal of both lobes and of the distal portion of the pars tuberalis and the infundibulum gave a positive Houssay effect and caused adrenal cortex atrophy. The magnitude of all three effects was correlated to the degree of encroachment on the hypophyseal stalk. From this it is concluded that the pars tuberalis is probably the crucial tissue concerned with the elaboration of the hormones involved in these effects.

INSULIN

Insulin increased and prolonged the oxygen uptake of skeletal muscle taken by biopsy from severe human diabetics (120). In two cases this effect was obtained without supplements; in two others only after the addition of dicarboxylic acid to the medium. Insulin was without effect on the respiration of muscle from non-diabetic patients.

Another *in vitro* effect of insulin has been described by Shipley & Humel (121). They found that addition of 100 γ of amorphous insulin to 1 cc. of rat serum containing liver slices of fed normal rats doubled the amount of sugar formed during two hours of aerobic incubation. This effect was not seen in liver slices of fasting rats or (with one exception) in liver slices of alloxan diabetic rats, possibly because of the low initial glycogen content and the relatively long incubation period. On the basis of total fermentable carbohydrate determination in the liver slices before and after incubation it was found that the liver of alloxan diabetic rats formed about three times more new carbohydrate than the livers of fasted rats. Insulin decreased new formation of carbohydrate by about 40 per cent in diabetic liver slices but had little effect in livers of fasted rats. There was no effect of insulin on the rate of ketone body production.

An *in vitro* effect of insulin which consists in the removal of an existing inhibition has been described by Price *et al.* (122, 123). The hexokinase reaction (glucose $+$ ATP $\rightarrow$ glucose-6-P $+$ ADP) represents the first step in the utilization of glucose in animal tissues. This reaction could be inhibited in tissue extracts by addition of an iso-

electric protein fraction of anterior pituitary, an inhibition which was greatly reinforced by adrenal cortex extract, and this inhibition could be released by insulin. When no inhibition was present, insulin had no effect on the hexokinase reaction. The conversion of glycogen to lactic acid in muscle extract was not inhibited by pituitary. Normal rats previously injected with pituitary or alloxan-diabetic rats (without injection) yielded muscle extracts which showed an inhibition of the hexokinase reaction, especially when adrenal cortex extract was added. While an inhibition was not obtained in every case in this type of experiment, probably because of difficulty of extraction of the pituitary factor and its very rapid inactivation by tissue extracts, insulin (in amounts of 50 γ per 2.5 cc. of reaction mixture) never failed to release an existing inhibition. These experiments would explain the much lower glucose utilization in depancreatized as compared to depancreatized, hypophysectomized animals and would indicate at least one point of action of insulin in carbohydrate metabolism.

Insulin increased the assimilation of glucose in artificially perfused limbs of cats, but had no effect on utilization of fructose even when the blood glucose was reduced to a low level (124). Although the plasma inorganic phosphate decreased after insulin, the ratio of phosphate to glucose disappearance in the perfused limb was too low to expect an accumulation of hexosemonophosphate (125). The P^{32} distribution in several phosphate fractions of the blood, including that of hexosemonophosphate, did not indicate an increased turnover after injection of insulin in guinea pigs (126). DeDuve & Hers (127) reported an increase in the hexosemonophosphate content of rabbit muscle following the injection of insulin and enough glucose to prevent hypoglycemia; this is contrary to results reported previously by the reviewers on cats and rats.

Changes in the respiratory quotient in normal men (128) and diabetic patients (129) have been studied following the injection of insulin; the response to insulin was generally a rise in R.Q. with relatively little change in the acid-base balance. Four cases of insulin-induced ketonuria have been reported (130).

Goldberg & Jefferies (131) reported a potentiation of insulin hypoglycemia in rabbits by subcutaneous injection of 5 mg. per kilo of sulfanylacetic acid. The survival time of eviscerated rats was markedly decreased by injection of insulin (132). Convulsive doses of insulin were found to be 10 to 100 times higher for birds than for mammals (133).

Experiments on Intact Animals and Changes in Blood Constituents

Survival times of rats fed exclusively on carbohydrates were studied by Richter *et al.* (134, 135, 136). When only glucose or only sucrose was fed to groups of female rats, they survived for thirty-seven days. Addition of thiamine lengthened the survival time to fifty-six and seventy-four days respectively. The rats developed vitamin A but no other vitamin deficiencies. "Dextri-maltose" was shown to contain enough thiamine to allow a survival time of eighty-five days, when it was fed as the sole food. Soulairac (137) gave mice fed on grain diet a choice between drinking water or 10 per cent sugar solution. Daily injections of insulin increased the consumption of glucose or sucrose but not of maltose solution.

Butyrate feeding did not lead to deposition of liver glycogen nor did it augment the glycogen when fed with glucose (138). Lackey & Bunde (139) also found no increase in the glycogen levels of liver and muscles when they fed butyrate to fasted rats. There was a slight increase in the heart glycogen and in blood ketones. Mannoheptulose did not form liver glycogen in rabbits (140). Wertheimer (141) found some deposition of glycogen in the fat tissue of rats during insulin hypoglycemia or after large doses of carbohydrate.

Handler (142) found that enzyme inhibitors such as cyanide, azide, malonate, fluoride, and iodoacetate, when injected in doses which caused death in rabbits in three to five hours, increased the blood levels of glucose, lactate, inorganic phosphate, and creatine. Only malonate caused a rise in acetoacetate. All the anesthetics studied by Stern *et al.* (143) depressed carbohydrate metabolism in men as judged from the blood sugar curves after glucose injection. Repeated injections of acetoacetate, β-hydroxybutyrate and pyruvate caused hyperglycemia in rabbits (144). Sokal (145) found that some liver extract preparations which are used therapeutically in the treatment of pernicious anemia contained a substance which caused hyperglycemia and glycosuria in men and rats.

Glucose tolerance tests were made in elderly persons (146, 147), after prolonged physical inactivity (148) and after gastroenterostomy and gastric resection (149). In all three cases the tolerance was diminished, as judged from the blood sugar curves. No difference in the blood sugar level was observed in twelve subjects who swam vigorously after a breakfast containing a high or a low percentage of carbo-

hydrate (150). Blood fructose determinations showed a diminished
tolerance to this sugar in acute hepatitis (151). An abnormally high
sugar content of the skin in relation to the blood sugar has been ob-
served in some skin diseases (152). Britton & Kline (153) reported
that administration of glucose prolonged the survival of rats exposed
to low barometric pressure. The speed of motor reactions in men in
acute starvation was found to parallel the blood sugar at values below
70 mg. per cent (154).

Blood lactate/pyruvate ratios in normal men were hardly changed
by a one hour walk, but were markedly increased twelve and thirty
minutes after a short period of "anaerobic" work; this was due to a
much greater rise in blood lactate than in pyruvate. Only in severe
thiamine deficiency was there a significant rise in resting lactate and
pyruvate levels associated with a decrease in the ratio (155). Simola
(156) reported a rise in blood α-ketoglutarate as well as pyruvate in
thiamine deficiency. Hemorrhage in dogs did not cause a change in the
lactate/pyruvate ratio (157). Himwich et al. (158) measured the
pyruvate concentration in the blood entering and leaving various
organs. Henschel et al. (159) found that the ingestion of 3 gm. of
citric acid had no effect on blood pyruvate levels in men.

Pyruvate metabolism has been reviewed by Stotz (160) and phlor-
hizin glycosuria by McKee & Hawkins (161).

METHODS

Somogyi (162) has described a new alkaline copper reagent for
the determination of sugars; it combines the advantages of several
of the old reagents and covers the range of 0.01 to 3 mg. of glucose.
The alkalinity (equimolecular mixture of di- and tribasic sodium phos-
phate) is high enough for the rapid oxidation of such sugars as malt-
ose. A new method of deproteinization which is equally serviceable
for whole blood and plasma and has other advantages over other
methods consists in the addition of adjusted solutions of zinc sulfate
and barium hydroxide. Schales & Schales (163) found the decrease
in extinction at 420 mμ of ferricyanide solution on heating with glu-
cose proportional to the amount of glucose present and have recom-
mended this as a simple method for the determination of blood sugar.

Colorimetric methods, based on the reaction with orcinol in the
presence of ferric chloride, for pentoses, pentosans (164), and various
nucleosides and nucleotides (165) have been described.

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DEPARTMENT OF PHARMACOLOGY
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FAT METABOLISM

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Reviews

Davison (1) reviews the literature on disturbances in lipoid metabolism in relation to the central nervous system. An excellent pathological discussion is included. Barach (2) reviews ketosis mainly from the clinical point of view. Mazza (3) reviews the literature on the dehydrogenases of higher fatty acids. The discussion centers about the occurrence, isolation and activity, mechanism of action, and the significance of these enzymes in the metabolism of higher animals. A consideration of the thermodynamics evolved is also included. Stadie (4) has also reviewed the current conceptions of the problem of intermediary metabolism of fatty acid. Cowgill (5) has reviewed the literature on the relative nutritive value of animal and vegetable fat. Lipoproteins are discussed by Chargaff (6), and lipocaic substances by Leites (7). The application of labeling agents to the study of phospholipid metabolism has been reviewed by Chaikoff (8). The role of the lipids in atherosclerosis is discussed by Hirsch & Weinhouse (9).

Methods

Forbes & Atkinson (10) describe a method for the quantitative fractionation of fatty acids, neutral fats, and sterols in feces. Mata (11) describes a rapid method for the determination of total lipids in 1 cc. of serum. The range of values in various clinical conditions was also determined. Marenzi & Cardini (12) also report on a colorimetric micromethod for the determination of total and unsaturated fatty acids in blood. Krainick & Muller (13) make use of the strongly colored fatty acid salts of colorless carbinol bases in a method for the photometric microdetermination of 0.05 to 0.5 mg. of fatty acids in blood or lymph. Hunter *et al.* (14) developed improved methods of extraction and titration of fatty acids for the microdetermination of tissue lipids. Sloot (15) also describes a method, based upon oxidation procedures, for the measurement of fat in tissue. Gortner (16) reported a comparison of three standard methods for the determination of phospholipids, viz., lipid P determination, Bloor's

method of oxidation of fatty acids from the acetone-insoluble lipids, and Bloor's direct oxidation of the intact phospholipids. MacLachlan (17) gives the details of a technique which eliminates error in the determination of the iodine number of whole phospholipids. Several papers are devoted to discussions of improvements in the microdetermination of ketone bodies in blood and tissues (18 to 21). Lipmann & Tuttle (22) describe a specific method for the determination of acyl phosphates. Histochemical reactions for lipid aldehydes and ketones (23) and sites of action of lipase (24) have been published. A micromethod for crotonic acid (25) and modified methods for the estimation of citric and acetoacetic acid in the presence of oxalacetic acid are described (26). A method for lipolytic activity of the duodenal contents is described (27).

ABSORPTION AND EXCRETION

Barcroft, McAnally & Phillipson (28, 29) studied the formation and absorption of acetic, propionic, and butyric acids from fermentation of cellulose in the alimentary canal of the sheep, horse, pig, and rabbit. Cardini & Serantes (30) followed the absorption of a fat ration consisting of olive oil and saturated fatty acids. The total fatty acids in the intestinal mucosa increase sharply while the phospholipids remain relatively unchanged. The unsaturated fatty acids increase more rapidly in the mucosa while in the blood the saturated acids increase first; these results indicate that saturated acids are retained in the mucosa for a lesser time than unsaturated acids. Frazer, Schulman & Stewart (31) report detailed studies of the rate of dispersion by the small intestines of ingested triglycerides. They also studied the effects of bile salts, oleic acid, soap, cholesterol, and glyceryl monostearate as emulsifying agents, either singly or in combination. Nguyen-Van-Thoai (32) studied the role of phosphatases in the transport of lipids. He reports that colchicine, fasting, phlorizin, or adrenal hormones have no significant effect on these phosphatases. Adrenalectomy slightly decreased the activity of the phosphatases of the intestines and kidneys. Zwemer & Wotten (33) studied fat excretion in the guinea pig kidney by histochemical methods and report that there is both excretion and reabsorption of fat by that organ. MacLachlan & Thacker (34) studied the effect of anoxia on fat absorption in rats. Within the range of oxygen pressures compatible with life there is no apparent effect. Roy & Sen (35) studied the effect of quinine on the absorption of fat. Their data indicate that neutral fat

absorption is diminished owing to lipase inactivation by the drug. Unlike monoiodoacetate, quinine does not prevent resynthesis of fat in the intestinal mucosa.

COMPOSITION AND DISTRIBUTION OF BODY FATS

General.—In studying the effect of a high fat diet upon the body composition of the albino rat, Wynn & Haldi (36) observed within one week a definite increase in the fat content and a decrease in the percentage of water and protein in the skin and body. These changes are qualitatively the same as those observed at the end of a seventy day period on the same diet. Shorland & De la Mare (37, 38) report studies on the C_{20} and unsaturated acids of pig back fat following periods of exclusive buttermilk feedings. Rathbun *et al.* (39) published interesting data on fifty normal guinea pigs including the measurement of the total body fat (ranging from 1.5 to 35.8 per cent of the body weight) and its specific gravity (ranging from 1.020 to 1.096). They find a high degree of correlation between their data and a theoretically derived equation so that it is possible to calculate the percentage of body fat from the specific gravity. In a second paper of this series Morales *et al.* (40) extend the theoretical discussion, and show how the relations may be applied to man. Gortner (41) presents valuable information on the lipid fractions of 438 pig fetuses during 70 per cent of the gestation period. Among the interesting findings reported are the following: The total lipid and lipid component ratios are relatively constant for a large part of the embryonic growth period; a considerable portion of nonphospholipid fatty acid is present in the unesterified form; fetal glycerides increase gradually beginning about the middle of the gestation period; however, at term only a minor part of total lipid substance is thus accounted for; and on a dry weight basis the phospholipid content is at a maximum in very young fetuses. Gatsanyuk (42) reports studies of the effect of antireticular cytotoxic serum on the amount of cholesterol, fatty acids, and water in the tissues of adult and senile rabbits. The free fatty acids in animal tissues have been investigated by Fairbairn (43). They average less than 2.2 mg. per gram of dry fat-free mouse tissue and 2.3 per cent of the acetone-soluble lipids of cat liver. Upon extirpation of the liver of rats or cats, the phospholipids present in the liver are rapidly hydrolyzed by intracellular phospholipases. Millican & Brown (44) studied the isolation and properties of some naturally occurring octadecenoic acids in a variety of animal and plant fats. Pace & Rathbun (45), discussing

the concept of a constant *milieu interieur*, present data on guinea pigs supporting the concept of a lean body mass relatively constant in gross chemical composition as determined by combined nitrogen and water content. Body fat acts merely as a "diluent."

Blood lipids.—Mazzoleni (46) studied the relation between the unsaturated blood fatty acids and the blood sugar. In normal man there is no significant change in the unsaturated fatty acids of the plasma following administration of glucose. In patients having diabetes or Basedow's disease, in contrast, there is a considerable decrease which is greatest at the time of the maximum blood sugar values. Friedlander, Chaikoff & Entenman (47) studied the effect of ingested choline on the turnover of plasma phospholipids. Using radioactive phosphorus as the labeling agent, they found that a single feeding of choline (300 mg. per kg. of body weight) accelerated phospholipid turnover in the plasma. This reflects the accelerated phospholipid activity in the liver which is known to follow choline ingestion. Since practically all phospholipids of the plasma are of the choline-containing type (48), it is impossible from this evidence to state whether or not choline also affects the noncholine-containing phospholipids of the liver.

Phospholipids in tissue.—Artom (49) has employed his method for ethanolamine and serine (50) to the determination of phosphatidyl ethanolamine and phosphatidyl serine in tissues. Data on the amount of these phospholipid fractions in some rat tissues and in human plasma in addition to the total and choline-containing phospholipids have been evaluated. Artom found in most tissues that the sum of the ethanolamine- and serine-containing phospholipids approximated the value for the noncholine-containing phospholipids. In the lung, kidney, and brain, however, ethanolamine- and serine-containing phospholipids together are greater than the amount of the noncholine-containing phospholipids. Serine-containing phospholipids have been found in all the materials analyzed: they represent only a small fraction of the total (and also noncholine-containing) phospholipids. Welch (51) has improved the method for the separation of the α and β-isomeric forms of lecithin and cephalin. She has applied it to the study of the phospholipids of the heart, liver, and brain of rats, cats, guinea pigs, and beef. She found in the liver and heart that β-cephalin and α-lecithin predominate. In the brain phospholipids, the α forms predominate. Taurog, Entenman & Chaikoff (52) studied the choline-containing and noncholine-containing phospholipids of plasma. From the data

on the mol ratio of choline to phosphorus, they concluded that practically all of the phospholipids are of the choline-containing type. Approximately 5 per cent of plasma phospholipids of man and dog contain no choline. Stewart & Sinclair (53) studied ricinoleic acid as a possible precursor of phospholipids of rats. The animals were fed for several weeks on a diet containing 8.4 per cent of castor oil. Only about 2 per cent of the fat intake was excreted in the feces. The distribution of ricinoleic acid in the tissues was calculated from a determination of the acetyl number; they found none in the phospholipids of the small intestines, liver, and muscle nor in the liver glycerides. However, the depot fat contained approximately 7 per cent of ricinoleic acid. That this hydroxy acid is readily metabolized is indicated by the fact that only 1 to 2 per cent of the ricinoleic acid absorbed was deposited as fat. Using tracer technique, Kaplan & Greenberg (54) have studied the distribution and turnover of acid-soluble phosphate of the liver as influenced by high fat, high protein, and high carbohydrate diet. The results are discussed in relation to the influence of antecedent diet upon the metabolic pattern of the liver, particularly with respect to the formation of energy-rich phosphate bonds.

Liver fat.—Williams *et al.* (55) report extensive studies on the lipid partition of isolated cell nuclei of dog and rat liver. They determined the cephalin, lecithin, sphingomyelin, free and combined cholesterols, cerebroside, and neutral fat in preparations from normal dogs, normal rats, and rats fed butter yellow. Comparable samples of the whole liver tissue were similarly analyzed. Of the essential total lipids 90 per cent are phospholipids and less than 5 per cent sphingomyelin. In the tumorous liver cells following butter yellow ingestion, there is a greatly reduced concentration of phospholipids and increased amounts of cholesterol esters. The content of neutral fat in the nuclei is surprisingly high. Cerebrosides, absent in the nuclei of normal rat or dog liver, are found in the nuclei from tumorous livers. The data indicate that the pathological derangement of the phospholipid content of the liver in the butter yellow tumor occurs primarily in the nucleus. The sources of the extra liver fat in various types of fatty livers were studied by Stetten & Salcedo (56) with the aid of deuterium. Two metabolic states were studied, viz., rats on an alipotropic diet, and mice injected with anterior pituitary substances. In choline deficiency impaired transportation of fatty acids from liver to depot was responsible for the fatty livers. Following the feeding of cystine, the fatty livers are attributable to an increased rate of fatty acid synthesis simi-

lar to the case of thiamine administration. In fasting mice, the fatty livers following anterior pituitary injection result from the excessive mobilization of depot fat with migration to the liver. This latter process is apparently the cause of human fatty livers as seen in routine autopsy materials. The effect on the liver fat of animals of diet high in glucose or sucrose prior to anesthesia by carbon tetrachloride is discussed by Dillard, Spence & Forbes (57).

Milk lipids.—Baldwin & Longenecker (58) found the fatty acid composition of cow colostrum to be very similar to that of butterfat. Gould (59) has studied the influence of pH, type of fat, and pancreatic extract upon lipolysis in homogenized milk. Hilditch and his co-workers (60 to 63) report detailed studies of the fatty acid composition of the goat, ewe, mare, and human milk.

Miscellaneous organs.—MacLachlan (64) placed mice on high fat diets and studied the lipid content of the lung. From his data he concluded that the lungs do not participate actively in fat metabolism. Remington & Harris (65) determined the degree of enlargement and edema, and also the iodine content of the thyroid of young rats placed upon diets containing high and low proportions of saturated and unsaturated fats. They concluded that the thyroid gland is not significantly related to the metabolism of fat. Kaufmann & Shaw (66) report further studies on the metabolism of the mammary gland of the cow. When the diet is comprised solely of carbohydrates, the lower fatty acids of milk are synthesized. The action appears to be indirect since blood glucose is not a precursor of the lower acids. Possibly fermentation of the carbohydrate in the rumen or a sparing action on the utilization of other body tissues may furnish the precursors. Patterson, Dumm & Richards (67) found that the lipids of the central nervous system of the honey bee are in general comparable to those of the brain of young vertebrates prior to the appearance of visible medullation.

FAT SYNTHESIS

By the use of deuterium, Bernhard & Bullet (68) established the fact that large amounts of fatty acids were synthesized by rats on high carbohydrate diets. They also report on lipid synthesis from protein (69), particularly as influenced by components of the B-vitamin group.

With the aid of deuterium, Stetten & Boxer (70) demonstrated in the rat that whereas only 3 per cent of the dietary glucose is converted to glycogen, at least ten times as much is used to synthesize

fatty acids. In the alloxan diabetic rat (71) the synthesis falls to about 5 per cent of the normal value. The urinary glucose recovered is approximately equal to this deficiency in fat synthesis. The author regards this failure of fat synthesis from carbohydrate to be a major metabolic defect in this disease. This failure of lipogenesis in the alloxan diabetic rat was also found (72) when alanine was the major dietary component. However, in phlorhizinized rats no such impairment was observed.

Dukler (73) reports that in rats fed protein the additional administration of the B-complex retards fat catabolism and accelerates its synthesis. Rats trained to eat at designated times were found to have a higher degree of saturation of liver fatty acids than the controls when fed glucose. Liver slices also showed higher respiratory quotient values in a glucose medium (74). Several papers have appeared dealing with the problem of fat synthesis by various microorganisms (75 to 79).

DIETARY FAT AND GROWTH

Boutwell *et al.* (80) reported further studies on the comparative values of butter fat, vegetable oils, and oleomargarine. They found that butter fat and lard produced a growth response equal to that following a diet of vegetable oil. With a mixed carbohydrate diet, vitamin fortified oleomargarine was equal in growth value to butter fat over a six weeks period; however, with lactose as the sole carbohydrate it was inferior. Harris, Sherman & Lockhart (81) studied the effects of hydroxy fatty acids, which are presumably constantly present in food fats and utilizable as sources of energy, on growth and development. They used three types of synthetic glycerides: tridihydroxystearyl glyceride, tri-trihydroxystearyl glyceride, and mono-dihydroxystearyl triglyceride. In the first case, compared to the controls, the fat showed superior gain in weight per gm. of food intake. In the second place the stearyl glycerides fed had little if any capacity to influence growth and development. The third type of hydroxy glyceride favorably affected the growth and development in proportion to the amount fed. In general, it was concluded that hydroxy fatty acid glycerides exert a favorable effect upon the growth and development of rats on diets already presumed to be adequate. Deuel *et al.* (82) in studies on the comparative value of fats, compared the growth rate and efficiency of conversion of various diets to tissue. Corn, cottonseed, olive, peanut, or soy bean oil, or margarine, when properly

supplemented with protein and vitamins was not inferior to butter fat in its effects upon the growth of weanling rats in a twelve week period. These findings refute the idea that butter fat possesses saturated fatty acids essential to growth which are not present in other fats. In a more extended series, Deuel, Movitt & Brown (83) came essentially to the same conclusion in support of the earlier work of Zialcita & Mitchell (84) on prematurely weaned rats. French & Elliott (85) studied the interrelation of calcium and fat utilization in the rat. Jack *et al.* (86) fractionated milk fats by precipitation from solvents at progressively lower temperatures and studied the growth response of weanling rats on the various fractions. They conclude that either the oleic acid content or the total unsaturation of the fats might be factors responsible for the differences in growth response. The same authors also report on the growth of young rats fed milk fat and synthetic glycerides as supplements to a fat-free diet (87). Deuel *et al.* (88) studied the growth and reproduction of rats over ten generations on Sherman diet B where the butter fat was replaced by margarine fat. They concluded that the vegetable fat can adequately replace butter fat.

FAT METABOLISM IN PATHOLOGICAL STATES

Lipoidosis.—Sperry (89) has reviewed the subject of the biochemistry of the lipoidoses in an interesting lecture. He concludes that "the underlying mechanism of the lipoidoses remains a complete mystery." An extensive bibliography is appended. Koberle (90) studied a series of cases of diffuse lipoidosis of the duodenum.

Neoplasia.—Hesselbach (91) reported that the incidence of spontaneous and induced tumors in mice and rats is influenced by the amount and kind of fat in the diet. Barboni (92) found in nine cases of carcinoma of the liver in cattle that the total fat in the tumor was lower, and the steroids were higher than in the healthy parts of the liver. Lawrason & Kirschbaum (93) studied in mice the effects of diets relatively high and low in fat respectively, on the appearance of spontaneous leukemia in the high leukemia F strain, and the carcinogenic induction of leukemia in strain Dba. They found that the incidence of leukemia was the same in both dietary groups, and that the induction of the disease was significantly delayed when the diet contained a relatively high percentage of fat.

György *et al.* (94) reported that linoleic acid, arachidonic acid, and oleic acid in descending effectiveness destroy butter yellow in experimental diets with the elimination of its effect on the liver. However,

a toxic substance producing anemia is formed. These experiments are used to explain why butter yellow together with Crisco or butter fat are procarcinogenic. Apparently the butter yellow is preserved in the diet and in the intestines because of the low supply of unsaturated fatty acids in the diet.

Miscellaneous conditions.—McArthur (95) studied the composition of the phosphatide-free lipid from the intima of atheromatous aorta. Govan (96) observed that fatty degeneration of the heart muscle could be produced by the administration of a high fat diet. Changes were produced more readily in well nourished than in lean animals. Andersen (97) in a study of the celiac syndrome compared two chemical methods with the microscopic estimates of fecal fats in normal children. She studied total fat, and the neutral fat and fatty acid fractions and discussed the clinical significance of each. Escudero, Mosto & Radice (98) reported upon the accumulation of the fat (lipopexia cells) of the lungs of the white rats when placed upon diets containing varying proportions of fat. Quantitative fractionations of the lipids of the serum and liver of seventy patients with diseases of the liver and bile ducts are reported by Man *et al.* (99).

ENZYMES CONCERNED WITH FAT METABOLISM

Cosby & Summer (100) describe a method for the purification of the soybean lipoxidase and adaptation of the qualitative carotene coloration test for assay. On the basis of new evidence Hummel & Mattill (101) discuss the possibility that lipoxidase is a dehydrogenase. Sullmann (102) presents detailed evidence on the inhibiting action of various substances on the oxygen uptake of linoleic acid, linolenic acid, and lecithin in the presence of soy bean lipoxidase. On the basis of spectrophotometric analysis, Holman & Burr (103) conclude that oleic acid, ethyl oleate, conjugated linoleic acid, linolelaidic acid, elaidolinolenic acid, and α-eleostearic acid are not substrates for lipoxidase. Linoleic acid, ethyl linoleate, linolenic acid, ethyl linolenate, and methyl arachidonate were found to be changed by the enzyme. El'yashkevich (104) published detailed studies of the lipase activity of the livers of rabbits, guinea pigs, and rats. Russian interest in this subject is further shown by the paper of Bondareva (105) on the effect of the oxidizers, iron, iodine, and hydrogen peroxide, and on liver lipase. Using synthetic triglycerides of fatty acid containing six to twelve carbons, Kraut, Weischer & Hügel (106) studied the rates of hydrolysis by pancreatic lipase. The rate was greater the smaller

the molecular weight of the triglyceride, but no appreciable difference was found between fatty acids with even or odd carbon atoms. Kabelitz (107), also working with synthetic fat containing both even and odd numbered fatty acids, observed no effect upon the serum lipase activity when these fats were fed unless the fats were administered in unusually large quantities. In studying the problem of the relation between the serum alkaline phosphatase and various diseases and sundry antecedent diets, Gould (108) reported that prolonged feeding of fat to rats is associated with increase in the serum phosphatase to very high levels. He believes this to be due to a quantitative alteration in the enzyme rather than to the action of an inhibiting or activating agent. The increased enzyme may be of intestinal rather than bone or kidney origin. In a continuation of their work on enzymes systems which require the sulfhydryl group for activity, Singer & Barron (109) studied enzymes which are concerned with fat metabolism. p-Chloromercuribenzoate, p-carboxyphenylarsine, iodoacetate, and iodoacetamide were used as inhibiting agents. The following enzymes appear to require the presence of a sulfhydryl group for activity: liver stearate oxidase, bacillus coli stearate and oleate oxidase, β-hydroxybutyric dehydrogenase, acetate oxidase, and pancreatic lipase. The enzyme in peanut oxidizing oleic acid appears to contain no essential sulfhydryl group. The authors point out that the enzymes which have now been demonstrated to contain essential sulfhydryl groups and which are concerned with carbohydrate, fat, and protein metabolism are considerable in number; a possible role of the ubiquitous glutathione is thereby made reasonable, namely, that it is concerned in cellular systems with the continuous reactivation of sulfhydryl enzymes which may be inactivated by oxidizing products of metabolism. Fontaine (110) confirms the conclusion of Lang that dog liver contains the higher fatty acid dehydrogenase which is active in the presence of coenzyme obtained from muscle. Muñoz & Stoppani (111) demonstrated the presence in cell-free extracts of rat kidney of a labile enzyme which oxidizes acetoacetic acid and β-hydroxybutyric acids. The supplements necessary for complete activity are inorganic phosphate, adenylpyrophosphate, cytochrome-c, and fumarate. Fluoride increases the rate of reaction. Apparently this system is different from any yet described. Lehninger (112) employed particulate but cell-free homogenates of liver to study the enzymatic oxidation of fatty acids C_4 to C_{18}. Adenosine polyphosphates are required for the oxidation, either as such or regenerated from phosphate donors, or by aerobic phos-

phorylations from the oxidation of pyruvate or fumarate. The author discusses the possible activation of fatty acids by the formation of acyl phosphate intermediates. The formation of lower glycerides during pancreatic lipolysis of fats and its significance in relation to the triple complex of bile salt-fatty acid-monoglyceride are discussed by Frazer & Sammons (113). Davies (114) has purified acetoacetic acid decarboxylase from *Cl. acetobutylicum* and studied its properties. It is stable at ordinary temperatures, specific for acetoacetic acid, and has a pH optimum at 5.0. The enzyme has a dissociable prosthetic group which is tentatively suggested to be riboflavin phosphate.

Fat Metabolism in Relation to Microorganisms

Kodicek & Worden (115) studied the effect of unsaturated fatty acids on *Lactobacillus helveticus* and other Gram-positive microorganisms. Oleic, linoleic, and linolenic acids cause complete inhibition of the growth and lactic acid production of *L. helveticus*. This is reversed by lecithin, cholesterol, calciferol lumisterol, α-tocopherol, and α-tocopherolacetate. A physicochemical interpretation of the phenomenon is suggested. Work on other Gram-positive bacteria is also included in the paper. Wood, Brown & Werkman (116) reported on the mechanism of butyl alcohol fermentation of *Cl. acetobutylicum* and *Cl. butylicum*, using labeled (C_{13}) acetic acid, butyric acids, and acetone as substrates. In the case of $CH_3 \cdot C^{13}OOH$, C^{13} was recovered in butyl alcohol, acetone, isopropyl alcohol, butyric acid, acetic acid, ethyl alcohol, and carbon dioxide. The butyl alcohol contained approximately 50 per cent of the added C^{13} in equal concentrations in the carbinol and β positions, which indicates that the molecule is synthesized from acetic acid or a derivative. The C^{13} in the carbon dioxide was about the same as in the acetone and isopropyl alcohol, which suggests that acetone is formed by decarboxylation of acetoacetic acid which is formed from the acetate. In the case of

$$CH_3 \cdot C^{13}H_2 \cdot CH_2 \cdot C^{13}COOH,$$

the C^{13} was recovered in butyl, ethyl, and isopropyl alcohol, and acetic acid and butyric acid. Eighty-five per cent of C^{13} was present in butyl alcohol in both the carbinol and β positions suggesting that butyric acid is a precursor of butyl alcohol. With acetone $CH_3 \cdot C^{13}O \cdot CH_3$, the C^{13} was recovered in isopropyl alcohol, indicating that acetone is an intermediate in its formation. Barker & Lipmann (117) made interesting observations on the lactic acid metabolism in propionic acid

bacteria which have significance in mammalian fat metabolism. Using dried preparations of *P. pentosaceum* they observed that the reduction of lactate to propionic acid is completely blocked by fluoride, the pyruvate fermentation being unaffected. Further when lactate reduction is so blocked there is no accumulation of lactate when pyruvate is the substrate. These results indicate that lactate is not an intermediate in pyruvate reduction and probably not even in the absence of fluoride. There is thus an analogy with butyrate which is known not to go through β-hydroxybutyric acid in its oxidation. Thus it is possible that a chain of reactions between keto and fatty acids not involving a hydroxy acid may be of significance in the succinic and "citric" acid cycles.

FAT METABOLISM IN RELATION TO VITAMINS

Baldwin, Longenecker & King (118) studied the effect of avitaminosis-C on fat metabolism in guinea pigs. In comparison with control animals there was no significant differences found in the gross amounts of liver, adrenal, or carcass lipids, phospholipid, or cholesterol content of livers, or cholesterol content of adrenals. In further studies, Baldwin & Longenecker (119) found no significant departure from normal in the acetone-soluble carcass lipid of ascorbutic guinea pigs. The component fatty acids of these lipids were found to be essentially the same as the controls. Giroud, Ratsimamanga & Chalopin (120) observed in ascorbutic guinea pigs inability to absorb olive oil from the intestinal tract. Large doses of adrenal cortical extract subcutaneously restored the absorption to normal. The authors suggest that the lack of vitamin C causes adrenal insufficiency. Booth, Henry & Kon (121) report a study of the antirachitic effect of fats on rats receiving high calcium-low phosphorus rachitogenic diets. The effect of the type of dietary fat upon the vitamin B requirement of rats on various carbohydrate diets is indicated by the work of Boutwell *et al.* (122). On medium levels of B vitamins, butter fat was superior to corn oil in growth promotion. This superiority could be eliminated by variations in the carbohydrate part of the diet, for example by glucose or galactose-glucose. On a lactose ration either of the two fats produced small growth, but this inferiority decreased as the level of the water soluble vitamin was increased. Possible explanations of these phenomena are discussed. Brown & Bloor (123) partitioned the fatty acids of butter into liquid and solid fractions and studied their effects upon the utilization of carotene from carrots when fed to rats. The storage of vita-

min A in the livers of the rats fed the liquid fat was greater than that in those fed the solid fatty acid diet. The same superiority of the liquid fatty acid diet was found in growth promotion. According to Waisman *et al.* (124), the interesting observation that thiamine-deficient mice are resistant to infection by poliomyelitis virus or Theiler's encephalomyelite virus is explained in part by the accumulation of pyruvate or other metabolites from incomplete carbohydrate breakdown. They studied the problem further by attempting to prevent the formation of pyruvic acid by high fat diets, but were unsuccessful because fat apparently has sparing action on thiamine in the case of mice. The presence of unsaturated fatty acid in the diet, according to Granados & Dam (125), is required for the depigmentation of the incisors in vitamin E-deficient rats. Apparently the phenomenon is related to some abnormal deposition or reaction of fat in the ameloblasts. The effect of chain length of the dietary fatty acid upon the fatty liver of choline deficiency was studied by Stetten & Salcedo (126). The even numbered fatty acids from butyric to stearic as ethyl esters were fed up to 35 per cent of the diet. As the chain length decreased from 16 to 14, fatty livers markedly increased, in contrast to the absence of severe fatty livers on fats containing twelve or less carbons. In studies on the utilization of thiamine in human subjects, Reinhold, Nicholson & Elsom (127) could find no evidence of a thiamine-sparing action of high fat diet. The amount of carbohydrate in the diet, however, was shown to be an important factor.

ESSENTIAL FATTY ACIDS

The nutritional deficiency in the albino mice resulting from diets devoid of fat is cured or prevented by the addition of lard to the diet (128). Bernhard, Steinhauser & Bullet (129), using deuterium, studied the question of synthesis of linoleic and linolenic acid in rats. The animals were on ether-extracted bread diet and in addition received heavy water. Analysis of the two fats in question obtained from the carcass fat showed no deuterium present in either case. These experiments confirmed their previous findings on the mouse that animal organisms cannot synthesize these essential fatty acids. Furthermore, supplementing a high carbohydrate diet with vitamin B_6 had no influence upon the results. An effect of tocopherol on essential fatty acid utilization is indicated by the experiments of Hove & Harris (130). They report that tocopherol greatly increases the effectiveness of methyl linolate in curing essential fatty acid deficiencies in rats.

When tocopherol but no essential fat is fed to essential fatty acid-deficient rats, there tends to be an increase in the severity of the deficiency. On the other hand, tocopherol fed to weanling rats on a fat-free diet delays the development of the essential fatty acid deficiency. The role of dietary fat and linoleic acid in the lactation of rats was reported by Loosli *et al.* (131). From these studies, linoleic acid does not appear to be a limiting factor in the growth of suckling rats.

LIPOTROPIC FACTORS

Investigating the possibility that choline and protein (or methionine) may be entirely responsible for the lipotropic action of lipocaic in preventing the dietary fatty livers of the white rat, Clark, Eilert & Dragstedt (132) found the addition of 0.5 per cent of methionine or 1.25 to 5.0 per cent of lipocaic to be effective, whereas 0.5 per cent cystine was not. Moreover, the composition of their lipocaic preparations led them to conclude that some constituent other than choline or methionine (or nonspecific protein action) was the responsible factor for its action. In dogs deprived of pancreatic juice by duct ligation or pancreatectomy, according to Chaikoff *et al.* (133), there occurs a fall in plasma choline which indicates a decrease in the choline-containing phospholipids; there is a concomitant development of fatty livers. The highly active pancreas fraction hitherto described (134) in daily doses of 60 mg. prevents the fall of plasma choline and the fatty livers in depancreatized dogs maintained on insulin. This action is not dependent upon the presence of choline as such in the diet. In further work on the pancreatic anti-fat factor, Chaikoff and his co-workers (135) find that methionine in the diet prevents the formation of fatty livers in depancreatized dogs. Apparently, then, the synthesis of choline from free methionine is not interrupted in the absence of the external pancreatic juice. It is suggested that the anti-fat factor is a proteolytic enzyme which makes available the methionine of the dietary protein for choline synthesis.

Further work on a beef liver fraction which in rats on a high carbohydrate, fat-free diet causes fatty livers is reported by McFarland & McHenry (136). These livers do not respond to choline, but the fat content is reduced by administration of inositol. In contrast, the fatty livers following biotin in place of the liver fraction are partially responsive to either lipotropic agent and completely so to the administration of both. No explanation for the difference in behavior of the two factors is at hand, nor is the nature of the constituent of the liver

fraction known. The earlier work of Engel was confirmed by Beveridge & Lucas (137) to the effect that the addition of corn oil to the diet increases the lipotropic action of choline but obliterates that of inositol. Presumably the essential fatty acids of corn oil are responsible. The liver phospholipids are uninfluenced by the removal of choline, inositol, corn oil, or "non-essential" fat from the diet, nor does any change result upon addition of these factors singly or in pairs. Beveridge *et al.* (138) discuss their work on the effect of dietary proteins and amino acids on liver fat in the light of the hypothesis that the amount of dietary methionine available for lipotropic action is limited to that portion not utilized by metabolic processes of apparently higher priority, such as growth and maintenance. The use of arachin, a globulin from peanuts, in the study of the lipotropic effects of methionine is reported (139). Brante (140) studied the phospholipids of liver, kidney, spleen, and muscle in choline and adrenal insufficiencies. His experiments did not support the view that phospholipid formation is concerned in the lipotropic action of choline.

Intermediary Metabolism

General.—Annau *et al.* (141) observed that the respiratory quotient of surviving rat liver was decreased in the presence of palmitate, stearate, and oleate. Supplementing these acids further with linoleate increased the respiratory quotient to almost 1, although by itself linoleate depressed the respiratory quotient. Linolenate itself raised the respiratory quotient, an effect opposed by the saturated 16 and 18 carbon acids. Lundback (142) reported on the respiratory quotient after administration of insulin and glucose to human subjects on a high- and a low-carbohydrate diet. From his results, he concluded that the blood sugar curve characteristic of "starvation diabetes" is attributable to low endogenous insulin or a predominance of fat metabolism in the liver. In rabbits, Markees (143) observed a hyperglycemia following butyrate injection which was abolished by the prior injection of insulin. His further results are discussed in the light of an interrelation between fat and carbohydrate metabolism. Lardy and his co-workers (144, 145, 146) reported extensive studies on the fat and carbohydrate metabolism of mammalian spermatozoa. He concludes that these organisms metabolize fats as well as carbohydrates through the isocitric cycle. Lipid oxidation results in phosphate esterification and the production presumably of adenosinetriphosphate, a clear indication that fat oxidation is coupled with phosphorylation and the production of

energy-rich phosphate bonds. Emmrich & Emmrich-Glaser (147), from feeding experiments on dogs, concluded that branched fatty acids of low molecular weight are not metabolized by omega oxidation. The isolation and behavior of the higher fatty aldehydes from tissue have been described by Anchel & Waelsch (148, 149). The relationship between glycogen content, fat content, and the respiratory quotient of liver slices from white rats is reported by Hindemith (150).

Miscellaneous fatty acids.—Upon feeding to man dicarboxylic fatty acids (C_6 to C_{11}) or their glycerides, Kabelitz (151) observed no increase in oxalic acid excretion. Adipic acid was found to be metabolized to a varying degree when fed to man (152). In the case of sebacic acid, Emmrich (153) found 30 to 46 per cent excreted unchanged in healthy normal subjects. Appel *et al.* (154) studied the growth of goats on various fatty acid diets including synthetic mixture of odd-carbon fatty acids. No significant differences were found with widely varying fat diets, but the interesting observation was made that the feeding of odd-carbon acids led to deposition of body fat containing up to 33 per cent of the total fat as odd-numbered acids (C_{13} to C_{17}). Lipmann & Perlmann (25) confirmed Quastel's opinion that β-hydroxybutyrate does not form from crotonic acid by the addition of water despite the existence of fumarase or aconitase in tissues. β-Hydroxybutyrate is the reduction product not the precursor of acetoacetate. They discuss the possibility of the formation of energy-rich phosphate bonds by reactions of the type:

$$-CH:CH- + H \cdot OPO_3H_2 = -C(OPO_3H_2):CH- + 2H$$
$$-C(OPO_3 \cdot H_2):CH- + H_2O = -C:O \cdot CH_2- + H_3PO_4$$
$$+ 11 \text{ Kgcal.}$$
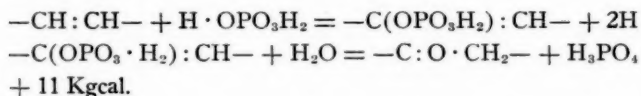
Studying the possible conversion of butyrate to carbohydrate, Deuel *et al.* (155) were unable to augment the liver glycogen in female rats fed butyrate four hours after glucose feeding. Unfasted male or female rats were similar to the controls in their response. Interesting observations on butyrate oxidation are reported by Leloir & Muñoz (156). Liver enzyme preparations (properly supplemented with adenylic acid, cytochrome-*c*, inorganic phosphate, magnesium, together with fumarate, succinate, malate, citrate, or glutamate) oxidize butyric acid vigorously. The above components of the Krebs cycle are potential precursors of phosphopyruvate, but the latter is active in increasing butyrate oxidation only in the presence of carbon dioxide. A phos-

phorylated C_4 compound is suggested as the possible "active" compound which reacts with butyrate to augment its oxidation. No intermediate formation of β-hydroxybutyrate occurs and the butyric acid is recovered almost entirely as acetoacetate. However, the oxygen and carbon dioxide are in excess of this reaction. In other words, some substance other than butyrate is oxidized concomitantly. There is either a coupled reaction or butyrate acts by maintaining the activity of some part of the system necessary for the oxidation of other substrates.

Acetoacetate.—Evidence continues to accumulate that the four carbon dicarboxylic acids are intermediaries in fatty acid oxidation. Wieland & Rosenthal (157), in general confirmation of the earlier work of Breusch, reported experiments indicating the condensation of acetoacetate and oxaloacetate to form a procitric acid. Upon hydrolysis acetic and citric acids result. The reaction was demonstrated in heart muscle and kidney tissue. The liver was inactive. By this mechanism, a common metabolic pathway for fats and carbohydrates would be established. The presence of barium ions is necessary. A further peculiarity is that the reaction, presumably nonoxidative, is diminished in nitrogen to one-tenth its value in oxygen. Weil-Malherbe (158) in similar experiments was unable to support the general conclusions of Wieland & Rosenthal. Krebs & Eggleston (159, 160) also disagree and in a preliminary paper determine all of the possible reaction products of acetoacetate and oxaloacetate, viz., acetoacetate, β-hydroxybutyrate, oxaloacetate, citrate, isocitrate, cis-aconitate, α-ketoglutarate, succinate, fumarate, malate, pyruvate, and lactate. All of the extra acetoacetate removed was fully accounted for as β-hydroxybutyrate and no hitherto unknown reactions were needed to explain the quantitative results. They concluded that oxaloacetate has no effect on the oxidative breakdown of acetoacetate, but, acting as a hydrogen acceptor, is itself converted to citric acid. Convincing evidence, however, comes from the experiments of Buchanan *et al.* (161). With kidney homogenates, they demonstrated C_{13}, originally present in acetate or acetoacetate, to be in α-ketoglutarate, succinate, or fumarate to a significant physiological degree. Carbon dioxide fixation was excluded. Citrate as an intermediate was also excluded since the C_{13} was always in the γ carboxyl of the ketoglutarate. Upon the basis of these findings they discuss the interrelations of carbohydrate and fat metabolism. Liver tissue converts carboxyl-labeled butyric acid to ketone bodies mainly by fission to 2-carbon fragments with

subsequent recombination, according to Medes *et al.* (162). About one-fourth of the total ketone formation is accounted for by direct oxidation. The absence of dilution of the isotopic butyric acid indicates that it is not an intermediate in the breakdown of tissue constituents.

Using washed particles from homogenized kidney cortex, Hunter & Leloir (26) studied the interaction of acetoacetate and oxaloacetate. Little acetoacetate was removed or citric acid formed unless α-ketoglutarate, glutamate, or glutathione was present. Both aerobically and anaerobically two molecules of extra citrate resulted from the disappearance of one molecule of acetoacetate. β-Hydroxybutyrate as a resultant of the interaction was excluded. It was suggested that *cis*-aconitate or an analogous compound is first formed. The need of ketoglutarate oxidation to make the reaction go is explained by assuming that the oxidation is coupled with the transformation of acetoacetate to an intermediate (possibly acetyl phosphate) reacting by condensation with oxaloacetate. The two mechanisms known to produce citrate, (*a*) pyruvate and oxaloacetate, and (*b*) acetoacetate + oxaloacetate with simultaneous oxidation of ketoglutarate, might thus be explained if one common intermediate is formed which is capable of condensing with oxaloacetate. Although the authors failed to show that acetyl phosphate was such an intermediate, they leave the question open. Similar views are voiced by Lehninger (112). Using his liver enzyme preparation (163) with saturated fatty acids (C_4 to C_{18}), he observed a synthesis of acetoacetate. In the additional presence of fumarate, however, this was diminished in favor of citrate formation. Acetoacetate formed citrate only in the presence of fumarate. He interprets his results to indicate that reactive two-carbon fragments are formed from: (*a*) pyruvate oxidation, (*b*) fatty acid oxidation in the liver, and (*c*) acetoacetate oxidation in the extrahepatic tissues. These condense with oxaloacetate to form "citrate."

Acetic acid.—Weinhouse *et al.* (164) studied the reaction of carboxyl labeled acetic acid in liver and kidney using C_{13} as the labeling agent. Acetoacetic acid was established to be an intermediate stage in acetate metabolism in liver. But other pathways could not be excluded. Approximately half of the increase of acetoacetic acid came from isotopic acetate. The distribution of C_{13} indicated that it is formed by coupling of two acetyl groups. In addition, the evidence indicated that acetate also forms from tissue constituents and is further oxidized in the kidney without appreciable accumulation of intermediaries. How-

ever, the possibility was suggested that C_4 dicarboxylic acids or other components of the Krebs cycle may be intermediates in the course of acetate oxidation in the kidney. Lorber, Lifson & Wood (165) studied the question of incorporation of acetate carbon into rat liver glycogen. Upon feeding isotopic acetate with C_{13} in both positions, they found excess C_{13} in liver glycogen at position 1,2,3,4,5,6 in contrast to the 3-4 position observed when carbon dioxide fixation is the sole mechanism. Hence, some pathway other than carbon dioxide fixation must be involved in the conversion of acetate to glycogen. Since long chained fatty acids are known to give rise to acetyl groups which may further be incorporated in amino acids presumably in equilibrium with carbohydrates, the interchange of carbon moieties of fats with those of carbohydrates by pathways other than carbon dioxide fixation appears established. Whether such mechanisms ever result in a net increase of carbohydrate from fat in the mammalian organism still remains an open question. The participation of acetate in the "citric" acid cycle was investigated by Rittenberg & Bloch (166) in an indirect way. Since ketoglutarate, oxaloacetate, etc., cannot be isolated from the intact animal, they examined glutamic and aspartic acids, which are in equilibrium with these components of the Krebs cycle. After feeding carboxyl-labeled (C_{13}) acetic acid to rats, the excess C_{13} in these amino acids indicated that acetate enters into the formation of carbohydrate intermediates by reactions exclusive of carbon dioxide fixation. These authors (167) also present evidence obtained by feeding acetic acid labeled by deuterium and heavy carbon showing that fatty acids are synthesized by condensation of acetic acid or of some derivative of it. The significant findings that the C_{13} excess in the carboxyl group of the saturated fatty acids isolated from liver and carcass of mice and rats is twice that of the average of all of the carbons is presumptive evidence that the labeled carbons are at alternate positions along the carbon chain. Thus a direct condensation of acetic acid into four carbon moieties which further condense into longer chains is indicated. Reaction schemes for fat synthesis including the role of pyruvic acid in similar condensations are discussed. The incorporation of acetate into cholesterol was also indicated by the finding that it contained both deuterium and C_{13}. Of the four reactions in which acetic acid is known to engage biochemically, viz., condensation to acetoacetate, formation of fatty acids, formation of cholesterol, and acetylization of foreign amines, Bloch & Rittenberg (168) employed the latter to estimate acetic acid formation in the rat. Isotopic acetic

acid with deuterium in the methyl group and C_{13} in the carboxyl group was used in feeding experiments. The compounds which were effectively acetylated were *p*-aminobenzoic acid, *d*- and *l*-phenylaminobutyric acids, and sulfanilamide. The first and third of these are acetylated only by acetic acid; to a minor extent, pyruvate is also effective in the case of phenylaminobutyric acid. The ratio of the two isotopes in the acetyl groups of the excreted amines was the same as that in the fed acetate indicating the stability *in vivo* of the carbon-hydrogen bond in acetic acid. Since acetic acid is the sole acetylating agent for the aromatic amines and the main one in the case of the amino acid, the observed isotopic dilutions in the excreted acetylated amines permitted an estimation of the daily endogenous formation of acetic acid. This was found to be approximately 15 to 20 mM per *d* per 100 gm. From isotopic analysis of cholesterol, following feeding of deuterio acetic acid, it was calculated that at least half of the carbon of the cholesterol was of acetate origin. Continuing their studies on ketone formation, Medes, Weinhouse & Floyd (169, 170) showed that ketone body formation is not exclusively a liver function, but may occur in tissues which metabolize acetate. Kidney oxidizes acetate so rapidly that no accumulation of ketone bodies results, but by studying the isotopic dilution in a mixture of isotopic acetate and normal non-isotopic acetoacetate when incubated with kidney slices they obtained evidence that acetate was condensed to acetoacetic acid in the kidney. The mechanism of formation is presumably the same as in the liver, i.e., by coupling of acetyl groups. Similar results were obtained with slices of heart muscle. The authors surmise that there are two pathways for acetate utilization: one through acetoacetic acid as in the liver and kidney, and the other, independent of ketone formation, occurring in the heart and perhaps also in the kidney and liver. Wieland *et al.* (171) reported detailed studies of the degradation of acetic acid, acetaldehyde, and citric acid in tissues. With respect to acetic acid, they concluded that it is not a degradation product of sugar in the muscles, but is utilized by the kidney through succinic acid. However, citric acid and α-ketoglutaric acids could not be detected as a product of metabolism of acetic acid. They also observed that the kidney converts acetaldehyde to acetic acid. The isolated beating heart of the rabbit utilizes perfused acetate at an approximate rate of 26 mg. per hr. No residual acetate was found at the end of the experimental period, nor was glycogen increased (172).

Acetyl phosphate.—Using a bacterial enzyme, Lipmann (173)

showed monoacetylphosphate to be the oxidation product of pyruvate. He also demonstrated the enzymatic transfer of phosphoryl groups between acetyl phosphate and adenylic acid and the reversibility of the reaction. From approximate measurements of the equilibrium constant, the free energy of the reaction was estimated to be −3.55 Kcal indicating that the bond energy of the (acetyl) carboxyl phosphate is some 25 per cent higher than the average of 12 Kcal of the energy-rich phosphate bond. Lipmann & Tuttle further report (174) on the condensation of acetyl phosphate with formate or carbon dioxide in bacterial extracts. Using radioactive phosphate, they showed a rapid interchange of phosphate between the acetyl compound and inorganic phosphate. They also estimate the equilibrium k for the condensation: acetyl phosphate-formate = pyruvate-phosphate to be of the order 0.01. Utter et al. (175) report on the oxidation of acetyl phosphate by *Micrococcus lysodeckticus*. Lipmann & Tuttle (176), using hydroxylamine, trap newly formed acyl phosphate as hydroxamic acid. With rat liver homogenates, acetic, butyric, octanoic, decanoic, and dodecanoic acids form hydroxyamic acids indicating the formation of acyl phosphate. The reaction occurs in air or nitrogen and without the presence of a phosphate donor.

Ketone body formation and utilization.—Shipley (177) found that liver slices from normal rats, either fasted or fed, produced ketone bodies, and that anterior pituitary extracts *in vitro* enhanced this formation. Diaphragm, kidney, spleen, omentum, and brain utilized ketone bodies. In addition to liver, kidney slices were found to produce new glucose abundantly. *In vitro* (178), the addition of insulin was without effect upon ketone formation by liver slices from normal or diabetic (alloxan) rats. Merlini (179) also reported the utilization of ketone bodies by isolated heart preparations, while Krauel & Gibson (180) showed no utilization of acetone by muscle or liver *in vitro*.

Ketosis.—Shaw and his co-workers have published an extensive series of papers on ketosis in cattle (181 to 187). The effect of overdoses of insulin in man producing ketosis is reported by Drey (188). The distribution of the ketone bodies in liver, muscle, and blood of healthy rabbits was studied by Somogyi & Stark (189). They concluded that only β-hydroxybutyrate is formed in the liver, acetoacetate being formed from it in the extrahepatic tissues. Somogyi (190) further reports on factors controlling ketonemia in normal subjects. A correlation between blood ketone levels and cardiac glycogen in alloxan diabetic rats was reported by Lackey (191). Other papers on

hunger ketosis (192), ketogenic activity of anterior pituitary (193), and the effect of glucose administration on diabetic acidosis (194) have been reported.

MISCELLANEOUS TOPICS

The relation of dietary fat to the diabetic state of alloxan treated rats was studied by Burn, Lewis & Kelsey (195). Upon abrupt change from a normal to a high fat diet (70 per cent of requirement) glycosuria disappeared, but ketonuria developed. Gradual substitution of the same diet resulted in a glycosuria without the concomitant ketonuria. The glycosuria returned after restoration of the normal diet, but became successively less after repeated periods on the high fat. Zialcita (196) was unable to establish any relationship in rats between fat feeding and galactose utilization. The effect of some 20 fatty acids and their derivatives upon the synthesis of acetylcholine by frog brain was reported by Torda & Wolff (197). Effects were variable and no generalizations were made.

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THE METABOLISM OF PROTEINS AND AMINO ACIDS

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General Aspects of Protein Synthesis

The problem of protein synthesis is of vital importance for an understanding of the metabolism of proteins. It is now clear that the mode of formation of a peptide bond *in vivo* is not the reverse of its hydrolysis. The metabolic formation of the peptide bond from amino acids involves an increase of free energy and must therefore be coupled to reactions yielding energy; the latter are presumably derived from the oxidation of carbohydrates. At various times it has been suggested that the peptide bond *in vivo* is formed by way of α-keto acids (1) or α-keto aldehydes (2). It is however unlikely that these compounds are directly employed. Evidence obtained by the isotope technique demonstrates that an α-amino group is present in the compounds employed in the synthesis of proteins. When ammonia labeled with N^{15} is administered to rats it is found that the isotope concentration in amino acids isolated from proteins is highest in glutamic acid (3). When, however, an amino acid labeled with N^{15} is fed, the amino acid having the highest isotope concentration in the protein is always the one fed (4, 5, 6). This clearly demonstrates that the amino acid fed has not first been deaminated and then reaminated during the coupling reaction. Had deamination occurred, the liberated nitrogen should have partitioned itself among the other amino acids in a manner similar to that found after the feeding of ammonia. In support of this view, the isotope concentrations in the other amino acids after the feeding of *d*-leucine, which must first be deaminated and reaminated to give *l*-leucine, approximate that found after the feeding of ammonia; the isotope concentration in the *l*-leucine of the protein is less than that in the glutamic acid (7). There is no direct evidence indicating that the carboxyl groups also are intact in the precursors of the peptide but it seems unnecessary to postulate the participation of aldehydes in the process. The organism can reduce carboxyl groups to aldehydes (8), but it is to be doubted that the reaction can be carried out at a rate sufficient to permit the very rapid synthesis of proteins which occurs in the living cell. It seems more likely that either the

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amino or the carboxyl group is activated prior to condensation with another amino acid or peptide. This activation might consist in the formation of N-phosphoryl amino acid from active phosphate associated with the oxidation of glucose (9). The reaction of such energy-rich substituted amino acids with the carboxyl group of other amino acids could yield peptides and phosphoric acid accompanied by a decrease of free energy. Similarly, transfer of an active phosphate to the carboxyl group would furnish an energetically feasible mechanism. The carboxyl-phosphate linkage as found in diphosphoglyceric acid (10) and in acetylphosphate (11) is significant in regard to the latter suggestion. The use of active phosphate groups by the organism as a mechanism for the transfer of energy is quite common.

Similar mechanisms can be postulated, employing other biologically available acids, for the activation of amino acids. The feeding of some unnatural amino acids results in their excretion as acetyl derivatives which like peptides are substituted amides. Study of the mechanism of acetylation of these amines has shown that both the aromatic amine, sulfanilamide, and the aliphatic amino acid, γ-phenyl-α-aminobutyric acid, can be acetylated by acetic acid, but that only the α-amino acid can be acetylated by alanine. The mechanism for this reaction presumably involves the oxidative deamination of alanine to pyruvic acid which is the active acetylating agent (12, 13, 14). The reaction does not involve conversion of pyruvate to acetate. These results show that the organism has a method for acetylating some α-amino acids by an oxidative exergonic reaction, a reaction similar to the nonenzymatic oxidative acetylation of choline with pyruvic acid described by Baer (15). If this reaction is but a special case of a general mechanism, then a plausible scheme can be formulated to account for the formation of peptide bonds.

$$\left.\begin{array}{l}\text{proteins}\\ \text{glucose}\end{array}\right\rangle \rightarrow CH_3 \cdot COCOOH \qquad (a)$$

$$R \cdot CHNH_2COOH + CH_3COCOOH \xrightarrow[-CO_2]{+O}$$

$$R \cdot CHNH(COCH_3) \cdot COOH \qquad (b)$$

$$R \cdot CH \cdot (COOH)NHCOCH_3 + HOOC \cdot CHNH_2R' \rightarrow$$
$$R \cdot CH(COOH)NH \cdot CO \cdot CHNH_2R' + CH_3COOH \qquad (c)$$
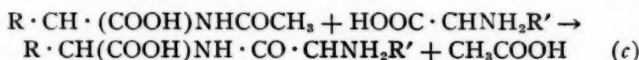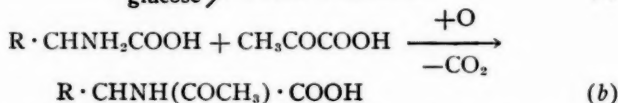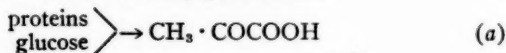
The peptide bond is formed in reaction (b). Reaction (c) is merely an exchange of the acetyl group by a substituted acid, the

amino acid.[1] Such mechanisms of course only partially explain the problem; specificity must be related to the action of suitable enzymes in reaction (c). While acetylation is not necessarily involved in the amination of keto acids as first suggested by Knoop, it may be involved in the synthesis of peptide bonds.

The isotope technique has shown that the cellular proteins are in a state of flux, being rapidly degraded and resynthesized. In the livers of rats, half the proteins are degraded and resynthesized in about seven days (6). There are two plausible mechanisms to account for this. The first envisages a complete hydrolytic cleavage of the protein molecule down to its constituent amino acids, while the second suggests that a single amino acid is removed from the polypeptide chain and replaced by an identical amino acid. During the replacement the two free ends may be held in the appropriate special configuration by secondary valence forces.

From the kinetic viewpoint the second hypothesis seems more attractive, but there is some evidence in favor of the first hypothesis. Heidelberger *et al.* (16) have actively immunized a rabbit to type III pneumococcus by injection of the appropriate antigen. After the type III antibody in the circulating serum had reached a maximal level, the rabbit was passively immunized to type I pneumococcus by transfer of immune serum. Glycine labeled with N^{15} was then added to the rabbit's diet. After three days the type I and type III antibody and a sample of serum proteins were isolated from the serum of this rabbit by the use of the appropriate specific polysaccharide. Both the serum proteins and the type III antibody produced by active immunization contained appreciable concentrations of N^{15}, proving that synthesis of these proteins had occurred. The type I antibody, however, contained no N^{15}. While our knowledge of the structures of the antibodies is incomplete, there is general agreement that the differences in the chemical structures of types I and III pneumococcus antibodies are small. If there were a mechanism which replaced amino acids in an otherwise intact protein, such a mechanism should operate on the type I as well as the type III antibody, since the mutual relationships of the amino acids over a large part of these antibodies must be quite similar. The fact that no labeled nitrogen is found in the type I antibody suggests that such a mechanism does not exist and that the process leading to the introduction of labeled amino acids in a pro-

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[1] This mechanism is being investigated by the reviewers in collaboration with Dr. K. Bloch.

tein is associated with a synthesis of the entire molecule. This argument is merely suggestive; further investigation of the kinetics of antibody formation may throw light on the subject.

PLASMA PROTEINS AND HEMOGLOBIN SYNTHESIS

The problem of blood protein synthesis has been actively pursued in several laboratories. Fine & Seligman (17, 18) have labeled the plasma proteins of dogs either by feeding amino acids prepared with radioactive sulfur or by brominating plasma proteins with radioactive bromine. Due to the difficulty of preparing normal proteins labeled with radioactive sulfur, they carried out most of their work with the unphysiological bromoproteins. Recently Fink and others (19) in Whipple's laboratory have prepared labeled plasma proteins by feeding dogs with lysine containing 66 per cent N^{15} in the ε-amino group. These labeled proteins were injected into dogs to replace plasma proteins removed by bleeding. The amount of this protein present in the circulatory system at any time was measured by determination of the N^{15} concentration of the nitrogen released on treatment of protein samples with nitrous acid. The protein nitrogen liberated by this reaction is derived principally from the ε-amino group of lysine. The loss of the labeled protein from the blood as indicated by the declining N^{15} concentration of the protein nitrogen so liberated is not logarithmic. In normal dogs half the labeled protein disappeared in twenty-six hours but the further loss of half of the remaining material took five days. The authors interpret the data as indicating a mixing within one day of the injected proteins with a pool of mobile tissue proteins. The pool is about as large as the amount of plasma proteins in the blood stream. The subsequent loss of labeled protein is the result of degradation and resynthesis of the plasma proteins.

The flow of proteins in and out of the blood stream is the same in shocked as in normal dogs. The half-life time for the synthesis of proteins in dogs is found by these authors to be of the same magnitude as in rats (20) and humans (21). It would have been of interest to have had the rate of excretion of N^{15} in the urines of these dogs, for from the data the authors give it is not possible to tell whether the injected protein had mixed with a pool of mobile tissue proteins or if half of the injected protein had suffered an irreversible change which rendered it unphysiological. This altered protein might have been hydrolysed to its constituent amino acids and metabolized in the same manner as if the protein had been given by mouth.

Miller *et al.* (22) found that hemoglobin or plasma proteins may be used effectively to supply the protein requirements of dogs since animals receiving these proteins remained in nitrogen balance. The data suggest that the prosthetic group of the hemoglobin is not reutilized and is excreted as bile pigment. Other investigators (23, 24, 25) have demonstrated the inadequacy of hemoglobin as a protein source for growing rats.

The addition to the diet of the ten amino acids essential for growth results in rapid production of plasma proteins (26) in dogs depleted by plasmapheresis. Omission of leucine or isoleucine results in a negative nitrogen balance and rapid loss of weight though plasma production may be temporarily maintained. Tryptophane deficiency causes an abrupt decline in plasma protein production. Histidine deficiency does not produce the marked effects shown by the amino acids mentioned above. Intravenous injection of aspartic or glutamic acids induces vomiting (26, 27). An unknown dietary factor seems to be required for optimal plasma protein production. It is found in liver and yeast (26). Similar results are reported by Ruegamer *et al.* (28) who found that additions of either milk or folic acid are required for maximal hemoglobin formation.

Robscheit-Robbins *et al.* have measured the maximum hemoglobin and plasma protein production under the stimulus of depletion (29). Under these acute conditions a dog can produce approximately one gram of hemoglobin and more than one gram of plasma proteins per kilo body weight per day. These figures are greater than the probable rate of synthesis involved in the continuous degradation and synthesis of blood proteins in the normal adult animal. Apparently depletion of the blood proteins is able to accelerate the normal rate of synthesis at which the organism prepares blood protein.

Orten & Orten (30) have tested the effect of individual amino acids on hemoglobin formation in rats made anemic by a low protein diet. No sustained increase in hemoglobin concentration was observed after addition to the diet of any one of ten amino acids essential for growth or of five amino acids, including glycine, which are not essential for growth. They reasonably conclude that no single amino acid can be regarded as a key in hemoglobin synthesis but rather that a combination of amino acids is necessary. These results are certainly consistent with recent views on the dynamic state of tissue proteins. Other studies on blood proteins may be found in the following papers (31 to 36).

DECARBOXYLATION AND TRANSAMINATION

In 1944 Gunsalus, Bellamy & Umbreit (37) reported that pyridoxal phosphate functions as a coenzyme for the *l*-tyrosine decarboxylase of *Streptococcus faecalis* R. It appeared at that time that pyridoxal phosphate differed from the codecarboxylase isolated from yeast by Gale & Epps (38) for the latter workers stated that their coenzyme did not contain phosphorus. However, subsequent work by Umbreit *et al.* (39, 40) and by Baddiley & Gale (41) showed that the differences concerning the nature of coenzymes previously postulated by the two groups are really nonexistent. Further evidence was provided for the identity of pyridoxal phosphate and the coenzyme or codecarboxylase of tyrosine decarboxylase by Umbreit *et al.* (39). These authors found that the activity of pyridoxal phosphate as a coenzyme for tyrosine decarboxylase, whether prepared enzymatically from pyridoxal and adenosinetriphosphate or chemically from pyridoxal and phosphoryl chloride, is very much the same as that found for the natural codecarboxylase. They also obtained evidence which strongly indicates that pyridoxal is a component of the natural codecarboxylase since the loss of activity of codecarboxylase occurring on storage at pH 7 and on boiling with 0.1 N hydrochloric acid for sixty minutes could be restored by the addition of adenosinetriphosphate to their assay system. Pyridoxal was the only known material which acted in this manner in their assay system. The reappearance of codecarboxylase activity after the addition of adenosinetriphosphate could readily be explained by the synthesis of codecarboxylase from the pyridoxal and the added adenosinetriphosphate. Baddiley & Gale (41) found on reanalysis that the codecarboxylase preparation of Gale & Epps (38) contained phosphorus. They also observed that dried organisms of *Streptococcus faecalis* grown on a pyridoxine-deficient medium will, on the addition of pyridoxal and adenosinetriphosphate, decarboxylate tyrosine. Codecarboxylase and synthetic pyridoxal phosphate are equally active as the coenzyme in the decarboxylation of tyrosine, lysine, arginine, and ornithine.

Codecarboxylase has been shown to be an ubiquitous substance (38), occurring in animal tissues, plant tissues, yeasts, and bacteria. It would appear that codecarboxylase (pyridoxal phosphate) is a required coenzyme for the decarboxylation of amino acids. However, Epps (42) has prepared an *l*-histidine decarboxylase and Taylor & Gale (43) have described a preparation of *l*-glutamic acid decarbox-

ylase from *Cl. welchii,* which could not be resolved by methods similar to that used for the resolution of other decarboxylases (38, 40, 43, 44) into an apoenzyme and coenzyme, nor could they find any codecarboxylase in boiled preparations. These authors conclude that the decarboxylases of histidine and glutamic acid isolated from *Cl. welchii* may not contain a codecarboxylase. On the other hand, Umbreit & Gunsalus (40) found that the glutamic acid decarboxylase from *Escherichia coli* may be resolved into apoenzyme and coenzyme and that the apoenzyme may be activated by synthetic pyridoxal phosphate. While the enzyme from *Cl. welchii* may not contain a coenzyme, it may be that the coenzyme for glutamic acid decarboxylase from *Cl. welchii* is more firmly linked to the apoenzyme than in the decarboxylase isolated from *Escherichia coli.*

Amino acid decarboxylases have been found in mammalian tissues. Blaschko (45), in a review of this subject, makes the suggestion, based on the finding of Stetten (46), that serine is converted into ethanolamine in the rat, that mammalian tissue contains a serine decarboxylase. However, it is not necessary to postulate the existence of a serine decarboxylase to explain this reaction. Shemin (47, 48) has recently shown that in the rat *l*-serine *in vivo* is converted into glycine by the splitting off of the β-carbon atom and that ethanolamine is not an intermediate. As Stetten (49) has shown that glycine is converted into ethanolamine, the conversion of serine to ethanolamine may proceed through glycine as an intermediate without the participation of the postulated serine decarboxylase.

The content of codecarboxylase in mammalian tissue is a function of the pyridoxal level in the diet (50). It is not as yet known, however, whether codecarboxylase is required for activity of mammalian decarboxylases.

Another function of the vitamin B_6 group in mammalian tissue has been established this year. In 1944 Snell (51) suggested, on the basis of his findings of enhanced growth of lactic acid bacteria after addition of pyridoxal and pyridoxamine, that these substances may be concerned in biological transamination. Later, Snell (52, 53) showed that on heating pyridoxal and glutamic acid a transamination occurred yielding pyridoxamine and keto-glutaric acid and that the reaction was reversible. The hypothesis that pyridoxal was concerned in biological transamination received support by the finding of Bellamy *et al.* (50) that resting cell suspensions of *Streptococcus faecalis* convert pyridoxamine into codecarboxylase if pyruvate is present.

The formation of the coenzyme is most readily explained by a transamination in which the amino group of the pyridoxamine is transferred to the pyruvate to yield alanine and pyridoxal. They suggested that this process may account for the replacement for bacterial growth of pyridoxine derivatives by alanine, as found by Snell (54). The former workers do not believe that alanine is converted to a member of the vitamin B_6 group, for alanine does not serve as a precursor of codecarboxylase for *Streptococcus faecalis* R, since they found that bacteria grown with alanine on B_6-deficient medium produced the tyrosine apoenzyme but not the complete enzyme (50). More concrete evidence for the function of codecarboxylase or pyridoxal phosphate in transamination has been furnished by Schlenk & Snell (55), Lichstein *et al.* (56), and by Green *et al.* (57). Schlenk & Snell reported that tissues from rats on a pyridoxine-deficient diet have a decreased ability to catalyze the transamination reaction of glutamic and oxaloacetic acids and that in some experiments the rate of transamination of deficient tissues could be stimulated by the addition of pyridoxal and adenosinetriphosphate. Lichstein *et al.* studied the relationship of pyridoxal to an active glutamic-aspartic transaminase in bacteria, a system previously described by Lichstein & Cohen (58). They found that cells of *Streptococcus faecalis* R, grown in a medium deficient in pyridoxal, were almost devoid of transaminase activity for the glutamate-aspartate system, and that the addition of synthetic pyridoxal phosphate stimulated the rate of transamination to a level comparable with that obtained for cells grown in a medium containing pyridoxal (56). Further, they partially resolved transaminase isolated from cells grown in a medium containing pyridoxal and found that the activity of the apoenzyme could be restored by the addition of pyridoxal phosphate. Cohen & Lichstein (59) reported that the cells of *Streptococcus faecalis,* whether grown in media free of or adequately supplied with pyridoxine, catalyzed the glutamate-aspartate transamination reaction equally well, though the cells differ in their ability to decarboxylate tyrosine. However, it was subsequently noted (56) that their medium contained small amounts of pyridoxine.

Green *et al.* (57) have isolated two purified transaminases from pig heart; one catalyzes the glutamate-aspartate system, the other the alanine-glutamate system. They have presented evidence for the existence of a coenzyme in their enzyme preparations. The properties of this coenzyme are paralleled by pyridoxal phosphate since it functions as a coenzyme for the "dopa" decarboxylase apoenzyme and

stimulates the growth of *Lactobacillus casei* in a pyridoxal-deficient medium.

The evidence points to pyridoxal phosphate as being the coenzyme of transaminase, though it has not as yet been established that transamination occurs by a process involving the cyclical amination and deamination of pyridoxal-pyridoxamine.

Kritsman reports that the formation of alanine in rat liver slices from pyruvic acid and ammonia proceeds rapidly in a bicarbonate buffer, but barely takes place in a phosphate buffer. Addition of oxaloacetate to the latter increases alanine synthesis (60, 61, 62). Kritsman believes that in the presence of bicarbonate, oxaloacetic acid is first formed from pyruvate and carbon dioxide, a reaction demonstrated by Wood & Werkman (63) and Evans & Slotin (64). The oxaloacetic acid is aminated and the aspartic acid formed transaminates with pyruvic acid to form alanine and oxaloacetic acid.

Braunshtein & Asarkh (65) suggest that many amino acids are deaminated indirectly. The amino acids first transaminate with α-keto-glutaric acid and the glutamic acid formed is then deaminated.

Amino Acids

Norleucine.—A sample of glycoleucine, later named norleucine, prepared by Thudichum (66) has been examined by Consden *et al.* (67). These authors subjected Thudichum's preparation to partition chromatography and the action of *l*-amino acid oxidase, and determined the melting point of the acetyl derivative. From their results they concluded that Thudichum's glycoleucine, or norleucine, is actually *dl*-leucine. Since Thudichum obtained the substance by alkaline hydrolysis of ox spinal cord, it is not surprising that racemization occurred. Consden *et al.* isolated *l*-leucine from an acid hydrolysis of ox spinal cord but could not find norleucine. The occurrence of norleucine in proteins has thus not as yet been demonstrated.

Isoleucine.—Devlin & Zittle (23) have shown that a diet containing human globin as the source of protein does not support growth in rats. However, on supplementing the globin with isoleucine, adequate growth was observed. Albanese (24) also found human hemoglobin to be deficient in isoleucine and *d*-isoleucine not to replace *l*-isoleucine for growth in rats. Orten *et al.* (25) also found that human or beef globin was inadequate for growth unless isoleucine was added to the diet. Their results show that removal of isoleucine from the diet leads to a severe anemia and final death of the rats. They suggest that

isoleucine is needed for normal hematopoiesis in the rat. However, it is not obvious that the anemia is specifically related to the lack of isoleucine, for protein synthesis in general cannot occur unless all the required amino acids are available.

Phenylalanine and tyrosine.—du Vigneaud and co-workers (68) have found that the addition of thienylalanine to a medium adequate for good growth of yeast causes a marked inhibition of growth which may be prevented by addition of phenylalanine. Thienylalanine was found to be incapable of supporting growth in lieu of either phenylalanine or methionine.

Darby *et al*. (69) have shown that liver slices of normal and scorbutic guinea pigs both produce a hydroxyphenyl compound from phenylalanine and metabolize tyrosine. These results together with those of Lan & Sealock (70) indicate that the defect in the metabolism of aromatic compounds by scorbutic guinea pigs is the inability of the deficient animals to oxidize the side chain of tyrosine. Kaser & Darby (71) showed that rats on a thiamine-free diet do not excrete any abnormal metabolic products of tyrosine or phenylalanine. Brugger (72) has observed that the close relatives of an imbecile did not excrete phenylpyruvic acid in the urine.

Tryptophane.—Albanese & Frankston (73) have found that human subjects excrete about 3 mg. of tryptophane per kilogram of body weight per day. Holt *et al*. (74) have shown that human subjects in negative nitrogen balance due to a tryptophane deficiency may be restored to nitrogen equilibrium by incorporation of 6 to 9 mg. of tryptophane per kilogram per day. The same group has demonstrated that acetyl-*d*-tryptophane, administered orally, is more readily metabolized than *d*-tryptophane (75).

Axelrod *et al*. (76), continuing their earlier studies, have shown that dogs on a pyridoxine-deficient diet excrete kynurenine and xanthurenic acid but no kynurenic acid after ingestion of single doses of 2 gm. of *dl*-tryptophane or 1.5 gm. of *l*-tryptophane. Animals receiving pyridoxine excrete kynurenine and kynurenic acid, but only traces of xanthurenic acid, after the administration of *dl*-tryptophane. The ingestion of tryptophane by pyridoxine-deficient dogs caused nausea and anorexia. Similar results were found by Miller & Baumann (77). These latter workers found that mice on a pyridoxine-deficient diet excrete xanthurenic acid in the urine in amounts that depended upon the tryptophane content of the diet. The survival time of pyridoxine-deficient mice depends on the amount of casein in the

diet; pyridoxine-deficient mice fed diets containing 60 per cent casein lived only one third as long as those whose diet contained 10 per cent casein. The xanthurenic acid excretion could be minimized by the addition of pyridoxine to the diet. The amount of pyridoxine required was proportional to the amount of casein present. The amount of excreted xanthurenic acid after intraperitoneal injection of *l*-tryptophane depended on the pyridoxine content of the diet.

Histidine.—Albanese *et al.* (78) have presented more evidence for the indispensability of histidine for the growth of rats and for weight maintenance in the adult rat. Holt & Albanese (79) have confirmed the work of Rose *et al.* (80) by showing that humans on a histidine-deficient diet do not go into a negative nitrogen balance. No definite conclusion is drawn concerning the essential nature of histidine in man. However, on a histidine-deficient diet a chromogen appears in the urine which disappears on the administration of histidine. Albanese *et al.* find *d*-histidine to be largely excreted by man (81).

Histidase, found by György & Röthler (82) and Edlbacher (83), has been shown to produce ammonia, glutamic acid, and probably formic acid (84) by action on histidine. Walker & Schmidt (85) studied the properties of histidase and its action on histidine, and believe that histidine is first converted into α-formamidino glutaric acid.

Holtz & Credner have presented further evidence for the conversion of histidine to histamine *in vivo* (85a).

Arginine.—According to Albanese *et al.* (86) *d*-arginine is utilized in man, and human liver arginase, in contrast to rat liver arginase, forms urea from *d*-arginine. They find that male adults excrete from 50 to 150 mg. of arginine daily. While human adults remain in nitrogen equilibrium-on a diet deficient in arginine the sperm count of these subjects decreases on an arginine-deficient diet (79). Two out of five cases of idiopathic hypospermia responded with a stimulation of spermatogenesis on being fed large amounts of arginine. The growing rat on an arginine-deficient diet develops marked anatomical changes in the testes.

Serine.—Artom & Fishman have described toxic symptoms and renal lesions caused by the administration of *dl*-serine by stomach tube. It is now found that only the *d*-isomer causes the toxic effects (87). The injurious effects of *dl*-serine are augmented on a diet low in protein and the vitamin B group; these effects are ameliorated by pyridoxine (90, 91).

Glycine.—Orten & Keller (92) have shown that rats on a low-protein diet become anemic and excrete less porphyrin than control rats. They therefore claim that amino acids serve as precursors of the porphyrins. This is undoubtedly true since porphyrins contain nitrogen, but the lack of porphyrin synthesis may merely be a reflection of the nonavailability of adequate amounts of amino acids for protein formation. Lack of globin may result in a decreased synthesis of porphyrin.

Shemin & Rittenberg have shown that glycine is the nitrogenous precursor of the protoporphyrin of hemoglobin in both man (93) and the rat (94). They fed glycine labeled with N^{15} and isolated hemin containing relatively high concentrations of N^{15}. Glutamic acid, proline, leucine, and ammonia are not directly employed for porphyrin synthesis. The data suggest that both carbon atoms of the glycine and the nitrogen atom are directly utilized for heme formation. The synthesis of a pyrrole from glycine and formylacetone has been described by Fischer & Fink (95).

The ability of the animal to synthesize the "non-essential" amino acids is well recognized. The mechanisms employed are understood for some of these, viz., tyrosine from phenylalanine (96), alanine, aspartic, and glutamic acids from keto acids derived from carbohydrates, arginine from ornithine (97), and cystine from serine (46, 98). Ornithine and proline (99, 100) are interconvertible but their precursor is unknown. The sources of the other non-essential amino acids, serine and glycine, have not previously been established. Shemin has recently shown that glycine is formed in the rat and guinea pig from serine (47) and that this conversion is specific for *l*-serine and proceeds by the splitting off of the β-carbon atom.

After injection of serine in which the carboxyl group was labeled with C^{13} and the amino group with N^{15} the glycine isolated contained N^{15} and C^{13} in the same ratio as the serine administered. These data suggest that the formation of glycine by the amination of glyoxylic acid, if it occurs at all, is a slow reaction. This is analogous to the finding that arginine is not formed by amination of its corresponding keto acid (101).

Ornithine-Proline.—Shemin & Rittenberg (101), after feeding glycine labeled with N^{15} to rats, isolated arginine and determined the N^{15} distribution in the ornithine moiety. The isotope concentrations in the α- and δ-amino groups were identical. A mechanism involving an intramolecular nitrogen shift was proposed to explain the equality

of N^{15} in both nitrogen atoms of ornithine and for the interconversion of proline and ornithine. The equality of isotopic concentrations in the α- and δ-amino groups in ornithine definitely demonstrates that arginine is not reversibly deaminated and aminated at an appreciable rate.

Lysine.—Neuberger & Webster (102) have continued the study of lysine deficiencies in the rat. They have found that lysine is required in the diet for the maintenance of body weight in the adult rat. While an adult rat seems to require a minimum of 16 mg. of lysine a growing rat requires about 100 mg. of lysine. Gillespie *et al.* (103) found that a lysine deficiency in rats of an initial weight of 110 to 140 gm. produced a mild anemia with a slightly greater reduction in hemoglobin than in red cells, a loss of protein from the liver, and a hypoproteinemia. The author suggests that the clinical picture is not specific for the amino acid deficiency but due to a general interference with protein synthesis.

It has been demonstrated by Doermann (104) that the growth of a mutant of Neurospora which requires lysine is inhibited by arginine. A molecular ratio of arginine to lysine of one reduces growth to one-half of that obtained in an arginine-free medium. If the ratio is doubled, growth is completely inhibited. Only the *l*-isomer is active.

Other amino acids.—Ames & Elvehjem (105) report that reduced glutathione may be oxidized in the presence of cytochrome-*c* by an enzyme system found in cell-free preparations of mouse kidney. They have also found that succino-oxidase may be inactivated by cystine (106). Albanese (107) suggests that *d*-cystine is partly utilized in man. Hamilton & Tarr (108) have determined the glutamine concentration of various tissues. The chemical characteristics and physiological role of glutamine have been reviewed by Archibald (109).

The participation of acetate in the synthesis of oxaloacetic and ketoglutaric acids has been established by three laboratories (110, 111, 112). A more detailed discussion of this subject will be found elsewhere (p. 193).

Two interesting reviews by Beadle (113, 114) have appeared dealing with the biochemical genetics of the metabolism of arginine, tryptophane, and tyrosine.

d-Amino acids.—Since the original paper of Kögl & Erxleben (115) there has been a continuing interest in the possible occurrence of *d*-amino acid in proteins. It is the reviewers' opinion that the evidence for the existence of *d*-amino acids in animal proteins is not con-

vincing. Wieland & Paul (116), employing the isotope dilution method, have found about 10 per cent of *l*-glutamic acid and about 0.5 per cent of *d*-glutamic acid in twenty-hour hydrolysates of Brown-Pearce tumors. This amount of *d*-glutamic acid probably arose by racemization during hydrolysis. Weil & Kuhn (117) claim to have found some *d*-leucine in the tail hair of old horses. The leucine was isolated by the Fischer ester-distillation procedure, a method in which racemization may occur.

Kögl *et al.* (118) have published another paper in support of their original thesis that tumor tissue contains extensive amounts of *d*-glutamic acid. In this article they describe the isotope dilution method, using deuterio glutamic acid as the labeled compound. The deuterio *l*-glutamic acid hydrochloride was prepared by adding 5 gm. of normal *l*-glutamic acid hydrochloride to 14.216 gm. of deuterio *dl*-glutamic acid hydrochloride containing 6.4 atom per cent excess deuterium. From this mixture 4.965 gm. of pure *l*-glutamic acid hydrochloride were obtained, which on analysis was found to contain 4.7 atom per cent excess deuterium. To the mother liquor, obtained after filtering off the *l*-glutamic acid, 5 gm. of normal *d*-glutamic acid hydrochloride were added, and 4.925 gm. of pure *d*-glutamic acid hydrochloride containing 4.95 atom per cent excess deuterium were isolated. This value, like that for the *l*-glutamic acid hydrochloride, is impossibly high. Whereas the entire mixture of the 5 gm. of normal *l*-glutamic acid hydrochloride and the 14.216 gm. of deuterio *dl*-glutamic acid hydrochloride would have an isotope concentration of 4.7 atom per cent excess deuterium

$$\left(\frac{14.216}{19.216} \times 6.4 = 4.7 \right),$$

the isotope concentration of the *l*-glutamic acid hydrochloride can only be 3.8 atom per cent excess. Only half of the 14.216 gm. of the deuterio *dl*-glutamic acid hydrochloride is of the *l*-configuration and mixes with the normal *l*-glutamic acid hydrochloride

$$\left(\frac{7.108}{12.108} \times 6.4 = 3.8 \right).$$

Similar considerations apply to the *d*-isomer. Kögl, Erxleben & Van Veersen do not seem to be aware of this for they state (p. 259) that deuterio *l*-glutamic acid can be obtained

.... indem man das Hydrochlorid der schwerem d,l-glutaminsaure mit der gleicher Gewichtsmenge des Hydrochlorids der "leichten" l- oder d-form mischt und aus der berechneten Menge verdunnter Salzsäure umkrystallisiert; so scheidet sich dann der überschüssize Antipode ab, dessen Deuterium gehalt natürlich nur halb so gross ist als jener der Ausgang verbindung.

The latter part of the statement is not correct. The deuterium concentration of the *l*-isomer will be but one-third that of the deuterio *dl*-glutamic acid hydrochloride, not one-half as they state. Moreover, the claim that over 4.92 gm. as pure *l*- and *d*-glutamic acid hydrochloride could be isolated after the successive addition of 5 gm. respectively of the *l*- and *d*-glutamic acid hydrochloride to 14 gm. of *dl*-glutamic acid hydrochloride is as astonishing as the claimed isolation of the free base lysine and of hydroxyglutamic acid in a previous paper (115). It is clear that either the isotope concentrations or the specific rotations of their *d*- and *l*-glutamic acid hydrochloride are in error.

A *d*-amino acid has been isolated from another antibiotic. One of the degradation products of penicillin is *d*-β-thiovaline (dimethylcysteine) (119).

AMINO ACID OXIDASES

Edlbacher and his collaborators have continued their studies of the enzymatic deamination of the amino acids. They find that not only *l*-glutamic acid but also *d*- and *l*-alanine, valine, leucine, and aspartic acid are oxidatively deaminated in brain tissue (120). These results are based upon ammonia production, the oxygen consumption not being considered significant by the authors.

The effect of various enzymatic poisons on kidney slices of guinea pig indicate to Edlbacher & Grauer the existence of more than one *l*-amino acid oxidase (121).

Edlbacher & Wiss (122) find that the *l*-amino acids exert an inhibitory influence on the action of *d*-amino acid oxidase when the enzyme exists in a relatively "high" concentration; conversely the *l*-amino acids activate the enzyme when the latter is present at "low" concentrations. In the latter case *l*-histidine has the most striking effect. These workers suggest that if the synthesis of amino acids is symmetrical, i.e., if *dl*-amino acids are formed, the activation of *d*-amino acid oxidase by *l*-amino acids may be important. There is, however, good evidence that the synthesis of amino acids *in vivo* is purely asymmetric (123).

Zeller *et al.* (124, 125) have found a water-soluble enzyme from the poison of *Vipera aspis* which oxidatively deaminates *l*-amino acids. The reaction liberates one mol of ammonia per atom of oxygen consumed. It differs in some of its properties from the *l*-amino acid oxidase of Green *et al.* (126, 127).

Hunter & Downs (128) have published a thorough study of the influence of the *l*-amino acids and some derivatives on the activity of arginase. In general all the *l*-amino acids inhibit the action of arginase at pH 8.4. This system is not inhibited by the *d*-amino acids, urea, native proteins, or amino acids in which the amino group is in other than the α- position. At any one concentration of arginine the "fractional activity," α, which is the ratio of the rate of hydrolysis of arginine in the presence of inhibitor to the rate in the absence of inhibitor, is described by the equation

$$\alpha = \frac{C}{I + C}$$

in which C is a constant characteristic for each amino acid and I is the concentration of the inhibitor. The mass of data in this paper is so great that no summary can do it justice.

UREA

Bach *et al.* (129) continue their attempt to demonstrate that a major part of urinary urea is formed by a mechanism other than that described by Krebs & Henseleit (97). In their experiments Bach and his associates attempt to show that compounds apparently necessary for urea formation, oxygen and ornithine, inhibit the action of arginase. If, indeed, the authors could prove that under normal physiological conditions arginase is inactive, then the Krebs-Henseleit cycle would have to be abandoned. But this they do not do. The concentrations of ornithine necessary to inhibit arginase are of the order of 1 per cent, a concentration which is unphysiological. There can be no doubt that arginase *in vivo* is actually active. If proof is necessary for this statement, it is found in the fact that the feeding to rats of arginine in which the amidine group is labeled with heavy nitrogen leads to the prompt excretion of almost all of the labeled nitrogen as urea (130). Secondly, there can be no doubt that the feeding of either ammonia or amino acids labeled with N^{15} results in the forma-

tion of arginine containing N^{15} in the amidine group (4, 5). Thirdly, after cessation of feeding of labeled glycine to rats the isotope concentrations in the amidine group of liver arginine and the excreted urea are nearly the same (6). The above data demonstrate beyond any doubt that in the intact animal the Krebs-Henseleit cycle must be operative; further it is quite likely that this general mechanism accounts for a major part of the urea formed. Those who claim that glutamine can form urea without interaction with the ornithine cycle have not offered convincing evidence.

Nutrition

Almquist & Grau (131) and Hegsted (132) have studied the growth of chicks on a diet in which the protein was replaced by amino acids. They find leucine, isoleucine, threonine, histidine, tryptophane, phenylalanine, and valine to be essential for growth. Almquist & Grau (133) find that not only glycine, but also glutamic acid is required in diets for optimal growth. This is strikingly different from the rat, which can synthesize glutamic acid at a rapid rate from keto-glutaric acid and ammonia. These authors found that cystine cannot compensate for a methionine deficiency even if high levels of choline are employed, and also that betaine and methionine can partially substitute for choline.

Hegsted *et al.* (134) have investigated the nutritive value of human plasma proteins in the rat and dog. For growing rats fibrin is a "high quality" protein, globulin is fair, and albumin is inadequate. The addition of isoleucine plus tryptophane to albumin gives good growth response. In dogs nitrogen balance can be maintained with 6 per cent albumin in the diet. Their results are in agreement with those of Cannon *et al.* (135).

Himsworth & Glynn (136) have continued their studies of the gross chemical changes occurring in the livers of rats on protein-deficient diets.

Infections, burns, and some operations give rise to a negative nitrogen balance. Madden & Clay (137) find that a sterile abscess produced by injection of turpentine produces in a normal dog a negative nitrogen balance, while it does not do so in a protein-depleted dog. Normal dogs on a high protein diet maintain a nitrogen balance. Grossman *et al.* (138) find that human patients with acute infectious diseases have a negative nitrogen balance in spite of high caloric and

high protein diets. Croft & Peters (139) find that addition of methionine to the diet of rats and humans suffering from burns abolishes the negative nitrogen balance.

In 1942 Woolley (140) found three unknown dietary factors essential for the guinea pig. Woolley & Sprince (141) find that these factors can be replaced by (a) folic acid, (b) casein plus cellulose or arginine, cystine, glycine, and cellulose, and (c) a concentrate obtained by the method used to prepare strepogenin. Strepogenin is as yet an unidentified growth factor required by certain bacteria (142). In properties it resembles a polypeptide. It seems to be present in pure proteins (143, 144), for partial enzymatic digests of insulin, trypsinogen, crystalline trypsin, chymotrypsinogen, chymotrypsin, ribonuclease, and other proteins are active in the *Lactobacillus casei* test. Considering their liver fraction to have unit activity, gelatine, dialyzed egg white, and salmine have a relative activity of less than 0.1, while crystalline trypsinogen and crystalline insulin have relative activities of 30 and 40 respectively. It is striking that crystalline trypsin has but 40 per cent of the activity of its precursor, crystalline trypsinogen. Strepogenin is destroyed by acid hydrolysis. The addition of casein and crystalline trypsinogen, proteins which are rich in strepogenin, or strepogenin concentrates increases the growth rate of mice on a diet in which the amino acids are supplied by an acid hydrolysate of casein fortified with cystine and tryptophane (145). Addition of egg white, a protein low in strepogenin, or hydrolyzed strepogenin concentrate was ineffective. The absence of this and possibly other essential peptides may account for the poor rate of growth of rats on a synthetic amino acid diet.

The role of the sulfur-containing amino acids in fatty livers is reported upon in several papers (146 to 150).

Bennett *et al.* (151) and Medes *et al.* (152) report on feeding rats a choline-free diet in which the only sulfur-containing amino acid was homocystine. On this diet some of the rats grew slowly, presumably synthesizing some methionine. Autopsies of rats showed nephrosis and evidence of healed kidney hemorrhages. These experiments seem to indicate an ability of the organism to synthesize slowly methyl groups (153). Du Vigneaud *et al.* (154) have directly demonstrated the synthesis of methyl groups by rats on a normal diet, i.e., one containing adequate amounts of biologically available methyl group. Deuterium oxide was injected into rats to increase their deuterium concentration and they were then given 4 per cent deuterium oxide

as drinking water for three weeks. The isotope concentrations found in the methyl groups of tissue choline indicate a synthesis of about 8 per cent during the three-week period. This small but not negligible synthesis may account for the growth of rats on a methyl-free diet.

The nutritional requirements of chicks are reviewed by Almquist (155), and of normal mice by Morris (156). A general discussion of the utilization of amino acids in therapy is given by Elman (157).

MISCELLANEOUS

Li *et al.* (158) have isolated a substance from the anterior lobe of ox pituitary which has the properties of the growth hormone. It causes the resumption of body growth and increase in the epiphyseal cartilage cells of the tibia in hypophysectomized rats. The material has the properties of typical protein. Its molecular weight is 44,250 and its isoelectric point 6.85. Its activity is destroyed by the action of trypsin and pepsin.

Borsook & Dubnoff (159) have shown that guanidoacetic acid can be methylated to creatine in rat liver slices by addition of *dl*-homocystine plus choline. These experiments demonstrate that homocystine can act as a methyl carrier from choline to guanidoacetic acid. It seems likely that methionine or a closely related compound is an intermediate.

Lustig *et al.* (160) injected into mice polypeptides prepared from yeast grown in a medium containing N^{15}. Relatively high concentrations of N^{15} were found in the skin after a short interval of time. Further investigations on the role of the skin in the metabolism of peptides seem desirable.

Nachmansohn & John (161) have studied the properties of the enzyme choline acetylase. They find that the dialyzed enzyme is activated by addition of *l*-glutamic acid, but not by *d*-glutamic acid. Strikingly, α-keto acids, including α-ketoglutaric acid, inhibit the enzyme. The effect of various compounds on the same enzyme system is described by Torda & Wolff (162).

Ussing (163, 164) has studied the reabsorption of glycine in the kidney of man and the permeability of erythrocytes to amino acids. Equilibrium between the extracellular fluids is established in two hours with the monoamino monocarboxylic acids; this process is very much slower with the dicarboxylic amino acids.

Field *et al.* (165) report that the feeding of the methylxanthines,

caffeine, theobromine, and theophylline, to dogs and to rabbits raises the level of the plasma fibrinogen.

Norris & Benoit (166, 167) have investigated the occurrence of trimethylamine and its oxide in various marine phylla. The oxide was generally found in crustacea. It appears to be of endogenous origin but its relationship to the metabolism of nitrogenous substances is obscure. There is a minute excretion of the oxide in the rat, which is increased by feeding but not by injecting choline; this suggests that the compound arises from bacterial action in the intestine.

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THE VITAMINS

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The accumulative effects of the war on vitamin research have been numerous and varied. Many scientific journals formerly published in the war zones have suspended publication. Because of difficulties of printing and transportation certain other publications have not been available. In spite of this situation, at least one thousand papers relating to different phases of vitamin research have been published during 1945. The current review is an attempt to present a representative cross section of those publications to which the present reviewers have had access. It is interesting to note that some laboratories have not deviated materially from the types of research projects on which they have published for several years. Many laboratories, however, have devoted their research facilities to problems directly or indirectly relating to the war effort.

One outstanding trend in the field of organic biochemistry has been the increased number of papers dealing with the synthesis of structural analogues, homologues, and derivatives of the vitamins. A chemical method for the conversion of β-carotene to vitamin A has been announced. The synthesis of folic acid has been reported, although actual research data and chemical procedures are not yet available.

Physiological researches have been characterized by additional emphasis on vitamin metabolites, studies on the physiological properties of structural analogues, antivitamins, vitamin-enzyme relationships, and on the biosynthesis of members of the water-soluble group.

The effect of the war has been particularly noticeable in the field of nutrition. Food shortages, rationing, lend-lease, and mass feeding problems stimulated governmental agencies to co-operate with industrial groups, academic institutions, and other research agencies to obtain more dependable information on vitamin requirements of human beings and of domestic livestock. The Food and Nutrition Board of the National Research Council has played an important role in much of this work. As a result of these studies and of nutrition surveys throughout the world, certain revisions of "vitamin allowances" have been announced by the Food and Nutrition Board.

Vitamin assay methods, especially microbiological methods, have received more than the usual amount of attention. Some progress has been made in developing improved techniques for determining physiological availability of vitamins in man.

Wartime necessity for more information concerning the vitamin content of natural and processed foods and the effect of cooking, processing, and storage of foods on vitamin stability and vitamin retention has been reflected in the 1945 vitamin research literature. Many of these researches are directly traceable to questions raised by Army, Navy, and various governmental agencies which had responsibility for the distribution of foods during the war period.

The following review of vitamin literature is presented from the standpoints of chemistry, physiology, nutrition, methodology, and the occurrence of vitamins in natural and processed foods.

CHEMICAL ASPECTS

Fat-Soluble Vitamins

During the past year chemical researches on the fat-soluble vitamins have not been numerous. As usual, the preparation of β-carotene of a high degree of purity still continues to be a problem. Devine and co-workers (1) were requested by the British Medical Research Council to prepare 20 gm. of β-carotene of highest possible purity. Details of purification are described. Ultraviolet absorption spectrum measurements on a fresh solution of the final products (in cyclohexane) revealed no inflection at 330 to 340 mμ. After five additional crystallizations there was little change in the spectrographic value. Final values obtained by these workers were higher than those recorded in recent literature. Deuel and co-workers (2) have measured the biological potencies of stereoisomers of α- and β-carotene. With an assigned biological value of 100 for *all-trans*-β-carotene, its isomer, *neo*-β-carotene U, was rated 38. *All-trans*-α-carotene had a potency of 53, while *neo*-α-carotene U had a rating of 13. However, Kemmerer & Fraps (3) report similar experiments in which the potency of *neo*-β-carotene U prepared from alfalfa was 25.

A series of papers have appeared from Karrer's laboratory dealing with the epoxides of the carotenes (4 to 7). Methods are described for the preparation of mono- and di-epoxides of β-carotene. These oxidation products are formed by breaking the double bond in the β-ionone ring, with the formation of an oxide in which oxygen

forms a bridge between the two adjacent carbons. Only one epoxide of α-carotene is possible owing to the presence of but one oxidizable β-ionone ring. Treatment with hydrochloric acid changes the epoxide to a furanoid structure, which possesses different chemical and physical properties. During this reaction some epoxide reverts to the original carotene structure, resulting in a mixture of carotene and the new furanoid compound. The authors claim to have found carotene epoxides in plant materials and the hypothesis is advanced that plant epoxides can be changed to furanoid compounds in plant tissues. No epoxides were found in animal tissues. β-Carotene epoxides possessed biological activity with rats while the furanoid form was inactive.

Hunter & Williams (8) claim to be the first to convert β-carotene to vitamin A by chemical methods. This is said to be accomplished by fission of the central double bond of the β-carotene molecule with hydrogen peroxide to give vitamin A aldehyde, which is subsequently reduced to vitamin A. The final product was identified by biological and physical methods.

Zechmeister & Polgar (9) have studied *cis-trans* isomerization of γ-carotene (lower melting form). They discuss eleven of the sixty-four possible isomers. Nash & Zscheile (10) describe the isolation of ζ-carotene from a special strain of tomatoes. Its absorption curve is similar to that of lycopene. Chalmers, Goodwin & Morton (11) have subjected vitamin A and one of its esters to the direct action of ionizing radiations. The ionic yield was practically unity in nonaqueous media and the effect was irreversible.

Robeson & Baxter (12) announced a new vitamin A (vitamer) isolated from fish liver oils. It differs from vitamin A in physical and chemical properties. It is thought to be a geometrical isomer of vitamin A, differing in the *cis-trans* configuration at the double bond nearest the hydroxyl group. The authors suggest a new system of nomenclature for the vitamers of vitamin A, based on the genus of fish in which they were first found.

Huber, Ewing & Kriger (13) have compiled valuable data relative to the ultraviolet absorption spectra of several nitrobenzoic acid esters of the provitamins of D_2 and D_3 as well as of similar esters of vitamins D_2 and D_3. These have been compared with the absorption spectra of corresponding free sterols and acids and also with the cyclohexyl esters of the same acids. Through the use of radioactive calcium and strontium, Greenberg (14) has studied the mineralization

of rat bones as affected by vitamin D. He confirms the theory that vitamin D promotes the absorption of calcium from the digestive tract but he feels that there is some evidence that vitamin D also exerts a direct effect on the deposition of calcium in the bones of rachitic animals.

Karrer & Kugler (15) describe the preparation of two new tocopherol derivatives in which the o-methyl groups are substituted by trimethylene and tetramethylene rings. The latter compounds were not biologically active. Two new homologues of α-tocopherol are also described (16) in which ethyl and propyl groups have been substituted for the 2-methyl group. These homologues were about one third as potent as α-tocopherol when tested with rats. α-Tocopherol is said to stimulate the synthesis of acetylcholine as measured by a modified Quastel method (17). The isolation and purification of d-γ-tocopherol from the vitamin E fraction of soybean oil has been described (18). This compound had a potency of 0.01 as compared with d-α-tocopherol. Tomarelli & György (19) believe that tocopherols act synergistically with rice bran extract in retarding oxidation of linoleic acid and, consequently, in preserving carotene. These conclusions were based on *in vitro* studies.

Chu (20) describes the synthesis of 4-(3′-methyl-4′-hydroxynaphthylazo)-benzenesulfonamide which, in preliminary tests, possessed antihemorrhagic activity almost equal to that of 2-methyl-1,4-naphthoquinone. Gaffron (21) states that vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) is present in all chloroplasts. Using the gas exchange method, he has studied the effect of K_1 derivatives on plant metabolism. Derivatives of the type of 2-methyl-1,4-naphthoquinone or the 3-oxy compound (phthiocol) accelerated respiration of *Chlorella* or *Scenedesmus,* similar to the action of dinitrophenols. They suggest that vitamin K_1, present in plants in concentrations of $10^{-3} M$, takes part in some metabolic reaction, as a catalyst or regulator.

WATER-SOLUBLE VITAMINS

Penney & Zilva (22) have confirmed the postulate that when dehydro-l-ascorbic acid undergoes mutarotation, 2:3-diketo-l-gulonic acid is formed. The calcium salt of this acid possessed no antiscorbutic protection for guinea pigs. The barium salt of the diketo acid was treated with hydriodic acid and the resulting l-ascorbic acid was found to be chemically and biologically identical with authentic

ascorbic acid. Koppanyi and co-workers (23) describe a new reaction of ascorbic acid with *dl*-alanine and hydrogen peroxide which forms a colored solution, and Bloch (24) describes a red color developed when cat gut is immersed in solutions of ascorbic acid. They believe that this is also a reaction characteristic of α-amino acids and ascorbic acid.

When water solutions of ascorbic acid are boiled in the presence of sucrose, tartaric, and citric acid, respectively (25), susceptibility of ascorbic acid to oxidation was somewhat decreased. Of the three compounds mentioned, sucrose exerted the greatest protective action. Mapson (26), in studying the oxidation of ascorbic acid, concluded that the action of the chlorine ion on the copper-catalyzed aerobic oxidation and on the reaction with hydrogen peroxide may be explained on the basis of its influence on the cupric-cuprous system. Bezssonoff & Leroux (27) submit evidence to support the theory that ascorbic acid plus copper plus iron constitute a monophenol peroxidase and that the vitamin and copper yield polyphenol peroxidase. They feel that ascorbic acid not only transports hydrogen but that it may also be responsible for a modification of the phenol molecule. Krukovsky and co-workers (28) add additional evidence to support the view that reduced ascorbic acid may play a role in causing tallowy flavor in milk during storage.

Buchman and co-workers (29, 30) have continued their work on thiamine analogues. They describe seven analogues in which the alcohol group in the 5-position on the thiazole ring was substituted by

(*a*) —H, (*b*) —C_2H_5, (*c*) —CH=CH_2, (*d*) —CHOH,

(*e*) —$CH_2 \cdot CH_2 \cdot CH_2OH$, (*f*) —$CH_2 \cdot CHOH \cdot CH_3$,

and (*g*) —CH_2OH.

None of these possessed biological activity when fed to rats. An eighth compound, 4-(5)-methyl-5-(4)-β-hydroxyethyl-imidazole, possessed no activity for pea roots or *Phycomyces blakesleeanum* (31). Emerson & Southwick (32) found that the 2-*n*-butylpyrimidine homologue of thiamine possessed antivitamin activity when rats were maintained on a suboptimal level of thiamine. An excess of thiamine, however, nullified the antivitamin effects of the homologue.

Sulfathiazole inhibits the carboxylase of yeast while 1 mol of cocarboxylase counteracts several thousand mols of sulfathiazole, according to Sevag, Shelburne & Mudd (33). It is suggested that cocarboxylase and sulfathiazole compete for the carboxylase protein.

Farrer (34, 35) has studied the chemical factors affecting the thermal destruction of thiamine and of cocarboxylase.

Furter and co-workers (36) synthesized two acetal and several succinate derivatives of riboflavin and tested them by biological (rat), microbiological, and fluorometric methods. Biological and microbiological activities of the succinates decreased in inverse relation to the extent of substitution. However, they were not equal, the microbiological activities being lower. These writers conclude that neither fluorescence nor microbiological response of *L. casei* is "necessarily a quantitative measure in the mammal for such flavins." Succinate solubilities increased with increasing substitutions.

Emerson, Wurtz & Johnson (37) found that the substitution of the ribityl group by the dulcityl radicle in riboflavin results in a compound possessing antivitamin properties when fed to rats. Stamberg & Theophilus (38) have added additional information concerning the destruction of riboflavin by photolysis. Forty per cent destruction was observed when bottles of milk were subjected to sunlight for two hours.

Ellinger (39) has published an excellent review of the literature relating to the metabolism of nicotinamide and the significance of urinary nicotinamide methochloride (F_2), while Racker & Krimsky (40) point out that nicotinamide may act to counteract the action of diphosphopyridine nucleotidase, confirming previous work of Mann & Quastel.

The direct role of pyridoxine in transamination reactions has been questioned by Cohen & Lichstein (41). Subsequent publications from the same laboratory (42, 43, 44) point out that pyridoxal phosphate serves as a coenzyme of at least three amino acid decarboxylases. Pyridoxal conversion to codecarboxylase is thought to be direct, while pyridoxamine must first be activated by pyruvate. The stability of pyridoxine, pyridoxamine, and pyridoxal has been studied by Cunningham & Snell (45). All three compounds are inactivated by light, although this occurs most rapidly in alkaline or in neutral solutions. Apparently the destructive effect does not depend upon the presence of oxygen. All are stable to light in 5 N mineral acid at 100°C. but all three compounds are destroyed rapidly by oxidizing agents. The properties of phosphorylated pyridoxal (synthetic codecarboxylase) have been described by Gunsalus and co-workers (46) and they suggest (47) that a derivative of pyridoxal acts as a coenzyme of tyrosine decarboxylase. Snell and co-workers (48, 49, 50) have also studied

pyridoxal and pyridoxamine with seventeen different organisms. They state that, on a molar basis, these compounds possess about equal activity for yeasts, molds, and rats. Responses of lactic acid bacteria varied from a few fold to several thousand fold when the aldehyde and amine were compared with pyridoxine. Doubt is expressed if these organisms can make use of pyridoxine. Using the information just described and employing differential assay techniques, they conclude that pyridoxal and pyridoxamine "constitute a considerable portion" of the total B_6 present in many natural materials. The Texas workers also observe that pyridoxal reacts with glutamic acid at elevated temperatures to form pyridoxamine and α-ketoglutaric acid. The reaction is reversible and may be regarded as a new example of transamination. Pyridoxal loses its growth-promoting properties when heated with histidine or tryptophane. These findings emphasize the difficulties involved in obtaining differential assays of pyridoxal and pyridoxamine in natural materials.

p-Aminobenzoic acid continues to be of interest in sulfa drug research. Goetchius & Lawrence (51) report that some new sulfonamides (sulfanilylanilides and benzenesulfonanilides) are not affected by the presence of p-aminobenzoic acid. Sevag, Henry & Richardson (52) state that sulfathiazole and sodium pyruvate compete for the active site of carboxylase. Presence or absence of inhibition depends on which compound gets to the active site first. They interpret their findings to mean that p-aminobenzoic acid behaves as an inhibitor of catalysis and not as a catalyst.

During 1945 a few papers have appeared dealing with the chemical synthesis of biotin and biotin analogues. Three of these from the laboratory of Schnider, Bourquin & Grüssner (53, 54, 55) deal with the synthesis of biotin and desthiobiotin compounds. Two researches from the laboratories of Merck & Company (56, 57) describe the methods for the synthesis of dl-biotin and discuss the stereochemical relationships of dl-biotin, dl-allobiotin, and dl-epi-allobiotin. Wood & du Vigneaud (58) also describe the synthesis of optically active desthiobiotin which, they state, appeared to be a mixture of two possible racemic forms. This mixture was only one-third as active as biotin in promoting yeast growth. A simple method of synthesis of dl-desthiobiotin is described by Duschinsky & Dolan (59), while Hofmann (60) has synthesized dl-oxybiotin (hexahydro-2-oxo-1-furo[3,4]imidazole-4-valeric acid). The total synthesis of 2,3,4,5-tetrahydro-biotin has been described by Cheney & Piening (61).

The chemico-biological aspects of biotin and desthiobiotin have also been considered. Tatum (62) suggests that desthiobiotin is a normal intermediate in the biosynthesis of biotin by *Penicillium chrysogenum,* while Winnick and co-workers (63) have studied the microbiological activity of *dl*-oxybiotin and related compounds. In no case was the activity of *dl*-oxybiotin equal to that of biotin when tested with three different organisms. Effect of structural modifications of the molecule on microbiological activity are also discussed. Similar studies on an oxygen analogue of biotin have been reported by Pilgrim and co-workers (64) and on *dl*-desthiobiotin by Rubin and co-workers (65).

The synthesis of a compound identical with the *L. casei* factor from liver has been announced (66). This member of the folic acid group was active for *L. casei, S. faecalis* R, and was also effective in promoting growth and hemoglobin formation in the chick. A crystalline product has also been isolated from the B_c conjugate obtained from yeast (67). The Wisconsin workers (68) have studied methods of liberating compounds of the folic acid group from foodstuffs. They conclude that Taka-diastase gave highest yields of folic acid in many foods, while the Laskowski enzyme from chicken pancreas worked well with a yeast extract and autoclaving at pH 4 for 12 hours at 15 pounds pressure gave best results with liver preparations. Mins & Laskowski (69) have described the preparation of a B_c conjugate which produces a *S. faecalis* and *S. lactis* R growth-stimulating factor from the inactive precursor in yeast. A new "conjugate unit" is suggested by these workers.

PHYSIOLOGICAL ASPECTS

HUMAN PHYSIOLOGY

Fat-soluble vitamins.—Further evidence has been advanced to support the hypothesis that, in human blood plasma, carotene and vitamin A exist in combination with protein, probably albumin (70). Attempts to increase the vitamin D content of human milk by feeding fish liver oil were found to be impractical. Polskin and co-workers (71) fed massive doses of vitamin D to eighteen mothers. When six of the women received as much as 480,000 I.U., the vitamin D content of the milk did not exceed 53 I.U. per quart. However, in a few other cases, milks were produced which ranged from 125 to 583 I.U.

per quart. In an eight-day balance study 1.30 per cent of the vitamin D ingested was excreted in the milk and only 6.90 per cent was accounted for in the stools.

Milhorat & Bartels (72) suggest that the utilization of tocopherols, in patients suffering from muscular dystrophy, is enhanced by simultaneous ingestion of inositol. This combination of factors was 40,000 times as effective in reducing creatinuria as wheat germ oil.

Water-soluble vitamins.—Mitchell and co-workers (73) have studied the excretion of ascorbic acid and dehydroascorbic acid in human sweat and urine under different environmental conditions. Twenty-four-hour excretions of dehydroascorbic acid in four human subjects averaged 0.8 mg. under "comfortable conditions" and 2.7 mg. under conditions which induced profuse sweating. Urinary excretion of ascorbic acid was highest under "hot-moist" conditions. It was concluded that losses of ascorbic acid and dehydroascorbic acid through the skin are not likely to affect the vitamin C requirement. Under hot-moist conditions urinary losses of ascorbic acid may be sufficiently large to affect the vitamin C requirement. Kyhos, Sevringhaus & Hagedorn (74) describe experiments in which large doses of ascorbic acid were fed to human subjects suspected of being deficient in vitamin C. Single doses of ascorbic acid, ranging from 100 to 500 mg., were efficient if the ascorbic acid content of the blood plasma was initially low. Some patients received as much as 1500 mg. of ascorbic acid, in one day without significant urinary loss and the plasma returned to high normal levels. In general, it was necessary to administer 1.5 to 2.8 grams of ascorbic acid to obtain saturation of some subjects. The authors emphasize the great variability in renal thresholds of human subjects. Johnson and co-workers at the Harvard Fatigue Laboratory (75) have published an excellent discussion of the controversy regarding vitamin balance tests. A careful study of sixteen human subjects led to the conclusion that intravenous and oral vitamin tolerance tests can be used with best results if urine samples are collected for one hour after the intravenous dose and during the second hour after the oral dose.

The availability of ascorbic acid by human beings has been studied by Hochberg, Melnick & Oser (76, 77). They could find no evidence for the destruction of dietary ascorbic acid in the digestive tract by ascorbic acid oxidases of ingested vegetables. *In vitro,* however, the vegetable oxidases carried oxidation beyond the dehydroascorbic acid stage when incubated at body temperature for six hours.

In another study, 7.0 mg. of copper were added to a homogenized mixture of the daily ration, with and without ascorbic acid, and tested by their human physiological availability method. It was concluded that copper had little or no effect on the availability of ingested ascorbic acid.

These workers have also studied the availability of thiamine in yeast by the physiological availability method (78). They conclude that thiamine in yeast is about 17 per cent available. This is lower than results obtained, in similar studies, by Parsons, Williamson & Johnson (79).

Melnick, Hochberg & Oser also studied the effect of ingested adsorbents on thiamine availability (80). Ingested kaolin did not interfere with thiamine utilization but fuller's earth lowered thiamine availability. These workers believe that *in vitro* tests with adsorbents give no index of human availability and that large doses of adsorbing agents are inadvisable unless adequate vitamin intakes are assured. The same authors (81) have reported *in vitro* studies on the destruction of thiamine in foods in the presence of thiaminase in fish products. Clam and herring products contained destructive thiaminase unless the enzyme destroyed by heating. Clams possessed the most destructive effect, while oysters, smoked carp, smoked salmon, and salmon caviar contained little, if any, thiaminase. In human availability tests, raw clams destroyed about 50 per cent of the dietary thiamine in the digestive tract. Some applications of thiaminase to thiamine methodology are also discussed.

Williamson & Parson (82) have studied the effect of high intakes of fiber (pineapple) on thiamine elimination in twelve human subjects. They conclude that a high-fiber intake tends to increase fecal elimination of thiamine. This is interpreted to mean that high-fiber diets tend to stimulate thiamine synthesis in the digestive tract, rather than interfere with absorption of dietary thiamine.

Hagedorn and co-workers (83) have studied riboflavin excretions in adult male prisoners. After ingestion of riboflavin, in food and in tablets, an increase in urinary riboflavin was noted. Retention varied from 17 to 80 per cent without any apparent reason for the wide fluctuations. When men received 0.5 mg. of riboflavin daily for a two-year period, no physical signs of riboflavin deficiency could be noted. Under these conditions the daily urinary output of riboflavin ranged from 0.5 to 0.12 mg.

Ellinger & Benesch (84) have submitted data, using eight human

subjects, which indicate that an appreciable amount of nicotinamide is produced in the digestive tract by biosynthesis. They measured urinary nicotinamide methochloride during and after administration of succinyl sulfathiazole (five cases) and of sulfathiazole (three cases). The former diminished the excretion of urinary nicotinamide methochloride by 60 per cent while only a small reduction was noted when the latter was administered. The succinyl compound did not interfere with the methylation of ingested nicotinamide and it was noted that urinary excretion of methylnicotinamide (nicotinamide methochloride) was lower in pellagrins than in normal people.

Another report from the same laboratory (85) describes results obtained with thirteen patients thought to be suffering from niacin deficiencies. The data were not conclusive but it was thought that urinary excretion of N^1-methylnicotinamide bore some relation to the clinical condition of the patient.

Johnson, Hamilton & Mitchell (86) have studied the effect of "comfortable" and "hot-moist" environments on the excretion of nicotinic acid, nicotinamide, nicotinuric acid, and N^1-methylnicotinamide by normal individuals. Ninety-four per cent of the total nicotinic acid and its metabolites, in the urine, were excreted as N^1-methylnicotinamide. Approximately 1.0 to 1.5 per cent was excreted as free nicotinic acid and from 3.5 to 4.5 per cent as nicotinamide. No nicotinuric acid was found in sweat or urine. It was concluded that the amount of nicotinic acid and its metabolites excreted in sweat is too small to affect the nicotinic acid requirement.

Perlzweig & Huff (87) suggest that there are, perhaps, two types of metabolic reactions involved in nicotinic acid metabolism. The first involves the methylation mechanism and the second, the subsequent conversion of F_2 to products as yet unknown. This, they suggest, may account for the inability of workers to correlate F_2 (N^1-methylnicotinamide) excretion with the state of niacin nutrition of the individual. Mickelson & Erickson (88) used two different analytical methods, with four groups of men, at different levels of niacin intake and found that it was impossible to note any difference in F_2 excretion. They conclude that F_2 excretion is not related to niacin intake.

The Illinois workers (89) have also studied the losses of pyridoxine and its metabolites in human sweat and urine. Under "hot-moist" conditions 50 per cent of the 8 mg. daily intake of pyridoxine was recovered in sweat and urine as pyridoxine and its metabolites. In urine over 85 per cent of the metabolites were present as 4-pyri-

doxic acid, while pyridoxine ranged from 4.0 to 4.5 per cent and pyridoxal (pseudopyridoxine) varied from 7.0 to 8.0 per cent. It was concluded that pyridoxine losses in sweat have no effect on the pyridoxine requirement.

These investigators have also made similar studies on pantothenic acid excretion (90). After the examination of eight-hour collections of sweat and twenty-four-hour collections of urine from five individuals subjected to "comfortable" and "hot-moist" conditions, it was stated that the data suggest but do not prove that the total excretion of pantothenic acid in urine and sweat may be increased under conditions of profuse sweating. Sarett (91) is of the opinion that the lactone and the dihydroxy moieties of pantothenic acid are not normal intermediates of pantothenic acid metabolism in man.

Gardner, Parsons & Peterson (92) have studied the metabolism of biotin in humans. Ten college women received low, moderate, and high biotin intakes. Total biotin output averaged 9.0, 3.0, and 1.5 times the dietary intake on low, moderate, and high biotin intakes, respectively, indicating biosynthesis of this factor in the digestive tract.

Johnson, Hamilton & Mitchell have also studied the excretion of folic acid (93) and choline (94) in sweat and urine. With *L. casei* as the test organism, it was found that five times as much folic acid was excreted in sweat under "hot-moist" conditions as was found in urine. When they employed the *S. lactis* assay, the amount of folic acid excreted in sweat exceeded that in the urine by six times. The choline excretion study, under similar conditions, indicated that only 0.7 to 1.5 per cent of the choline intake was excreted as choline and that "hot-moist" conditions did not increase choline excretion. The amount of choline found in undiluted sweat ranged from 2.7 to 15.30 µg. per 100 cc.

Moore, Bierbaum, Welch & Wright (95) describe clinical case reports in which the synthetic *L. casei* factor (folic acid) was used in treating pernicious anemia. They conclude that this preparation has antipernicious anemia activity, but they warn against the conclusion that it is identical with the natural liver principle.

ANIMAL PHYSIOLOGY

Fat-soluble vitamins.—Oser & Melnick (96) have compared bioassay data with colorimetric data on several plant foods. They conclude that, on the average, 1 µg. of crude carotene (free from xanthophyll and lycopene) as determined colorimetrically, was equal to 1

U.S.P. unit of vitamin A. However, this was not true for fruit products like apricot or peach purees in which the crude carotene values underestimated the biological values.

Moore & Cotter (97) fed low-carotene rations to dairy cattle and concluded that a low-carotene intake does not affect ascorbic acid synthesis to the extent of altering breeding efficiency. Lord (98) describes a two-year study of the vitamin A content of butterfat and sera from seventy pure-bred Ayrshire cows subjected to stall feeding and pasture feeding. Average values for total vitamin A in butterfat were 31 I.U. per gram when pasture fed and 15 I.U. per gram when stall fed. Twenty-eight per cent of the units in the "pasture butterfat" were attributed to carotene while carotene furnished 20 per cent of the potency of the "stall fed butterfat." In May and June carotene made a maximum contribution of 34 per cent. Serum carotene and vitamin A in pasture-fed animals were 1.35 mg. per 100 ml. and 150 I.U. per 100 ml. while in stall-fed cows the values were 0.23 mg. per 100 ml. and 31 I.U. per 100 ml., respectively.

Slanetz & Scharf (99) believe that soybean lecithin influences storage of vitamin A in the rat, and they postulate the existence of an unknown factor in soybean lecithin.

Sherman and co-workers (100) studied the storage of vitamin A in the liver of the rat on diets containing variable amounts of vitamin A. Rats receiving 3.0 I.U. of vitamin A per gram of dry food or 0.8 I.U. per Calorie stored negligible amounts of the vitamin in the liver. Liver storage increased with increased levels of dietary vitamin A. The efficiency of storage was of the order of 10 to 30 per cent of the total vitamin A ingested. In a second paper from the same laboratory (101), it is concluded that 3 I.U. per gram of dry food is not optimal but is near the minimum limit of adequacy. A similar study by Callison & Knowles (102) indicates that no measurable stores of vitamin A could be found in rats receiving less than 50 to 80 I.U. of vitamin A per kg. of body weight. This is about four times the minimum requirement of the rat. They suggest that the daily requirement of the rat for vitamin A is really higher than 20 I.U. per kg. body weight per day.

Pavcek & Elvehjem (103) studied telang livers from beef cattle afflicted with telangiectasis. These livers were toxic to rats and the toxicity was found to be due to an abnormally high content of vitamin A. Kligler & Guggenheim (104) damaged the liver of rats by causing enteric infection with *Salmonella typhimurium*. Infected rats

stored vitamin A in the same amount as noninfected controls. However, infected animals were not able to transform dietary carotene into vitamin A effectively. A second paper from the same laboratory (105) describes the effect of similar infections with the same organism on starved and vitamin A-deficient rats and mice. Hypovitaminotic rats were no more susceptible to the infection than were the controls. Avitaminotic rats and mice were more susceptible to the infection. A study by means of the paired feeding method led to the conclusion that starvation and not vitamin A deficiency was responsible for the increased susceptibility to *Salmonella typhimurium.*

Friedenwald, Buschke & Morris (106) report that in rats the speed of the mitotic cycle is reduced in vitamin A deficiency and that the mitotic rate per 1,000 basal cells is reduced by 30 per cent.

A series of five papers by Weinmann & Schour have appeared which deal with histological studies of rat teeth. The first paper (107) presents a study of the histological changes in the teeth of rats which have subsisted on a rachitogenic diet for fifty-six days. It was found that enamel formation and calcification were normal, while dentin formation was retarded. Enamel-covered dentin was more severely affected than cementum-covered dentin. The second paper (108) shows that, in rachitic rats, new bone formation proceeds at a normal rate but persists as osteoid tissue, which eventually undergoes resorption. In the third paper (109) they describe the effect of injecting normal and rachitic rats with parathyroid hormone. Osteoblastic activity was stimulated in all animals when doses were small but progressive osteoclasis occurred when doses were large. Osteoid tissue of normal rat teeth was more resistant to absorption than that of rachitic teeth. The hormone did not cause resorption of hard dental structures (enamel, dentin, and cementum). The fourth paper (110) involved a fasting study and a study of the effect of vitamin D_1 on histological changes in teeth of rachitic rats. Fasting, after rickets had developed, caused the same reparative changes in dentin as were caused by vitamin D_1. The layer of intermediate dentin which disappears during rickets redevelops in the basal third of the enamel-covered dentin. When phosphates were injected (111) osteoid tissues began to calcify on the second day after injection. Hyaline tissue, formed by degeneration of connective tissue, started to calcify immediately. Tissues which are formed last tend to calcify first. Reed & Reed (112) submit evidence to show that vitamin D exerts a peripheral catalytic action on osseous tissues, independent of its possible effect on calcium and phosphorus absorption.

McChesney (113) finds that chicken tissues inactivate vitamins D_2 and D_3, the former being inactivated approximately 50 per cent faster than the latter. This, he suggests, may explain why D_2 is less effective than D_3 for the chick.

Blood pressure studies on normal and vitamin E deficient rats led Telford, Swegart & Schoene (114) to conclude that vitamin E deficiency tends to cause a reduction in systolic blood pressure. Hove, Hickman & Harris (115) found that natural mixed tocopherols increased the survival time of rats to anoxia, when rats received a low fat, tocopherol-free diet. When the fat content of the diet was increased, survival time to anoxia was reduced.

Continuing his researches on the action of structural analogues of the various metabolites, Woolley (116) has shown that dl-α-tocopherol quinone caused hemorrhages in the reproductive systems of pregnant mice and resorption termination of pregnancy analogous to vitamin E deficiency. No effects were noted on nonpregnant mice. The above effects could not be remedied by administering α-tocopherol, but vitamin K (2-methyl-1,4-naphthoquinone) did prevent the harmful effects of dl-α-tocopherol quinone, which Woolley regards as a structural analogue of vitamins E and K.

Mason & Emmel (117) call attention to a characteristic intracellular pigment in smooth and skeletal muscles of vitamin E deficient rats. They suggest that this inert, yellowish brown, iron-free pigment is an abnormal metabolite or that it is a normal metabolite having a transitory existence in vitamin E deficient tissues.

Granados & Dam (118, 119) state that depigmentation of the incisor teeth of vitamin E deficient rats requires the presence of fat, presumably unsaturated fatty acids.

Lyons (120) believes that there is a thiol-vitamin K relationship in blood clotting. He postulates the liberation of thiol groups from the fibrinogen molecule, which oxidize and unite to form disulfide linkages on adjacent molecules. He believes that vitamin K may play its part by being a prosthetic part of one of the thrombin components or that it converts a protein fraction into an oxidation-reduction system with a potential in the vicinity of vitamin K.

Water-soluble vitamins.—The epinephrine content of the adrenal glands is increased in scorbutic guinea pigs, according to Banerjee (121). Langwill, King & MacLeod (122) found that the pH of urine tends to increase when rats are subjected to anoxia and that the ascorbic acid concentration of the urine tends to decrease as the pH increases.

Vavich, Dutcher & Guerrant (123) maintained a Holstein cow on an ascorbic acid-low ration for a period of fifteen months, during which time she gave birth to a normal calf. Plasma ascorbic acid usually fluctuated between 0.30 to 0.45 mg. per 100 ml., while the urinary excretion of ascorbic acid ranged from 14.1 to 97.8 mg. per 24 hours. Large doses of ascorbic acid increased the excretion of ascorbic acid very slightly and only temporarily. *In vivo* and *in vitro* studies showed rapid destruction of ascorbic acid in the rumen contents, due, in a large measure, to the action of microorganisms. When the cow subsisted on the ascorbic acid-low ration, the amount of ascorbic acid excreted in the milk and urine exceeded the ascorbic acid intake, adding further evidence for the synthesis of ascorbic acid by the bovine.

Lundquist & Phillips (124) found that the ascorbic acid content of bovine plasma may be increased by feeding chlorobutanol at the rate of 5 gm. daily. Symptoms of acute and chronic ascorbic acid deficiencies in the rhesus monkey have been described by Shaw, Phillips & Elvehjem (125).

Animal researches involving thiamine have been relatively numerous. Westerfeld & Doisy (126) were unable to show that alcohol increases the thiamine requirement of pigeons. In fact, these authors state that isocaloric substitutions of alcohol for fats and carbohydrates delayed the onset of acute thiamine deficiency symptoms. Kaser & Darby (127) maintain that thiamine deficiency in the rat does not result in the development of a defect in the metabolism of phenylalanine. Using the rat as the experimental animal, Hegsted, McKibbin & Stare (128) have shown that atabrine displays a thiamine-sparing effect.

Emerson & Obermeyer (129) found that thiamine-deficient rats excreted feces which were as rich in thiamine as those obtained from rats which received up to 50 µg. of thiamine daily. Fecal thiamine was largely in the form of cocarboxylase, especially in the latter stages of depletion. This fecal thiamine was found not to be available when feces were fed to thiamine-deficient rats.

Electrocardiographic abnormalities in thiamine-deficient rats have been described by Hundley and co-workers (130). These include bradycardia, auricular fibrillation, and other abnormalities which can be corrected by thiamine administration. Similar studies have been made on thiamine-deficient cats by Toman and co-workers (131). They found that electrocardiographic changes are of two types, i.e.

(a) disorders of rate, regularity, and impulse, which yield to thiamine treatment, and (b) changes in the ventricular complex, which respond much more slowly to treatment.

Griffiths (132) has produced a selectively bred strain of rats which are characterized by a high incidence of epileptiform seizures (audiogenic fits) when subjected to high frequency auditory stimulations. Supplements of thiamine in the drinking water tended to lower the incidence of audiogenic fits in eight of eleven animals, while four controls, without thiamine, showed no reduction in epileptiform seizures.

Shock & Sebrell (133), working with frog muscle, found that the addition of thiamine to the perfusion liquid caused increased work output. Optimal results were obtained when the thiamine concentration reached 0.001 mM. per liter. Cocarboxylase also increased work output. Paul and co-workers (134) have observed that the thiamine content of the skin, in rapidly healing wounds, is approximately double that of normal skin.

The thiamine content of rat blood parallels the thiamine content of rat tissues and both of these reflect the dietary intake of thiamine, according to Greenberg & Rinehart (135). Pence, Miller, Dutcher & Thorp (136) found that pig blood is normally about twice as rich in thiamine as human blood. These writers also call attention to the fact that the thiamine content of pig blood is closely related to the thiamine content of pig muscle and that both of these are related to levels of thiamine intake.

Biosynthesis of riboflavin and other B vitamins has been studied by Schweigert et al. at Wisconsin (137). These workers used normal and cecectomized rats on diets containing different types of carbohydrates and with and without the addition of sulfaguanidine and sulfasuxidine. Sulfaguanidine exerted antibacterial action in the digestive tracts of normal and cecectomized animals. Rats receiving sucrose, as the sole source of carbohydrate, did not seem to depend on the cecum for synthesis of the required factors. Riboflavin synthesis, on lactose diets, was decreased by cecectomy or by feeding sulfasuxidine, and similar results were obtained with dextrin, but not with sucrose. About twice as much riboflavin was excreted as was ingested when high levels of riboflavin were fed and about ten times as much was excreted when only 56 μg. were ingested.

Obermeyer, Wurtz & Emerson (138) have fed rats diets containing varying amounts of riboflavin. Fecal riboflavin remained constant for forty days, regardless of the level of riboflavin intake. The total

output of this vitamin was dependent on the quantity of feces voided. When the caloric intake was cut 50 per cent, less riboflavin was voided than by *ad libitum* controls.

Sure (139) has conducted riboflavin balance studies with rats which received this vitamin in the form of dried liver-concentrates and yeast. Increased fecal eliminations of riboflavin from liver-concentrate diets could be ascribed to intestinal synthesis but the fecal riboflavin from yeast diets was ascribed, at least in part, to lack of intestinal absorption.

Pearson & Luecke (140) state that nicotinic acid is synthesized by the horse and that trigonelline and N^1-methylnicotinamide are not the principal metabolic end-products in the horse, as they are in man, dog, and rat. Najjar, Hall & Deal (141), using operative procedures on the dog, conclude that the *in vivo* liver is capable of methylating nicotinamide to an appreciable degree. This was not true of the kidney. Woolley (142) has tested the ketone analogue (3-acetylpyridine) on mice which are not ordinarily susceptible to nicotinic acid deficiency. When two or more mg. of the analogue were fed, Woolley noted typical niacin-deficiency symptoms. Additions of nicotinic acid or its amide prevented the symptoms. The analogue possessed no marked bacteriostatic or bacteriocidal properties. When some bacterial inhibition was observed, the effect could not be counteracted by nicotinic acid.

Pyridoxine deficiency in rats brings about a latent erythropoietic inadequacy which is indicated by impairment in the rate of red blood cell regeneration after hemorrhage, according to Kornberg, Tabor & Sebrell (143). Reid, Huffman & Duncan (144) have produced experimental poikilocytosis in the bovine by maintaining open rumen fistulas. Since yeast products and pyridoxine were effective in treating the disease, they conclude that the disturbance of normal rumen synthesis of certain vitamins may have been the causative factor.

The paths of excretion of pantothenic acid by the dog have been studied by Silber (145). Urinary excretions, after doses of calcium pantothenate, were in inverse relation to the amount of food ingested with the vitamin. Much pantothenic acid appeared in the feces when food intakes were large, due to lack of absorption. None was found in the feces when calcium pantothenate was injected parenterally.

Carter and co-workers (146) describe two types of nutritional anemia. The first is caused by a lack of pantothenic acid and is characterized by a number of blood abnormalities, including a fall in hemo-

globin values and in polymorphonuclear leucocytes. The origin of the other type of anemia has not been ascertained.

Several types of animals have been used as subjects for biotin research during the past year. Emerson (147) points out that rats respond to synthetic and natural biotin but this was not true for *l*-biotin or *dl*-allobiotin. In another paper from the same laboratory (148) it is stated that succinylsulfathiazole failed to aggravate induced biotin deficiency in rats. Biotin is essential for successful gestation of rats and for the birth of viable young, according to Kennedy & Palmer (149) while Pizzolato & Beard (150) maintain that biotin is essential for the maintenance of the normal level of muscle creatine in rats.

Biotin deficiency of the monkey (*Macaca mulatta*) has been studied by Waisman, McCall & Elvehjem (151). Loss of hair was the principal sign of deficiency, which could be cured and prevented by biotin feeding.

Smith & Lasater (152) have produced a progressive paralysis in dogs on a basal ration containing all known members of the B-complex except biotin. Recovery was accomplished with doses of biotin as small as 100 µg. per kg. body weight.

Ott (153) found that synthetic biotin was as effective as natural biotin for the chick, while Hegsted & Stare (154) report that the biotin requirements of the duck are similar to those of the chick. Hofmann *et al.* (155) call attention to the fact that oxybiotin is unique inasmuch as it acts like biotin for the rat and chick. It is to be noted that this is the first known compound having activity for higher animals comparable with that of biotin.

Folic acid has also received considerable attention during the past year. Wright and co-workers (156) found that folic acid in milk powder is available to rats but that it is not available to test microorganisms, thereby explaining the reason for differences in assay values obtained by these methods. Leuchtenberger *et al.* (157) describe experiments with mice in which some evidence is afforded to indicate that folic acid (5 µg. daily by injection) caused complete regression of spontaneous breast cancers in thirty-eight among eightynine mice. Fewer tumors developed in treated mice as compared with controls.

Schweigert *et al.* (158) fed rats a basal ration containing dextrin plus sulfathiazole. Livers from these rats were lower in vitamin B_c than livers of controls. When liver supplements were fed, the B_c con-

tent of livers was increased five to nine times. Krehl & Elvehjem (159) state that previous variations in the response of dogs to nicotinic acid can now be explained on the basis of fluctuations in folic acid, the presence of which is essential for satisfactory response to nicotinic acid.

Day, Mims & Totter (160) have studied the relationship between vitamin M and the *L. casei* factor for the monkey. Vitamin M-deficient monkeys responded to the *L. casei* factor, indicating that these fractions of the folic acid group may be identical.

Hertz (161) believes that there is a quantitative relationship between dietary folic acid and stilbestrol in the metabolism of the chick. Campbell and co-workers (162) have shown that the crystalline B_c factor prevents macrocytic anemia, leucopenia, and thrombopenia in chicks during the first four weeks of life. Turkey poults require vitamin B_c, according to reports by Richardson, Hogan & Kempster (163). B_c (or folic acid)-deficiency in young turkeys is characterized by cervical paralysis but anemia is not marked.

Chaikoff, Entenman & Montgomery (164) advance the hypothesis that a substance exists in the pancreas which is active in maintaining the level of circulating choline and that this "external secretion" is independent of the dietary choline. These workers believe that this substance is related to the fall in plasma choline in the development of fatty livers. Luecke & Pearson (165) have fed as much as 40 gm. of choline per day for six days to sheep and dogs. Urinary nitrogen accounted for virtually all the choline ingested and no increase in the choline content of liver, kidney, or plasma could be noted.

PLANT PHYSIOLOGY

Wynd & Noggle (166) report that the amount of available soil nitrogen is a factor in affecting the accumulation of carotene in immature leaves of the oat plant. Teng-Yi (167) concludes that nickel and zinc have a physiological function in the promotion of carotene formation in peas. Tanner and co-workers (168) believe that trace elements are essential for riboflavin synthesis by *Candida* yeasts.

The synthesis of *p*-aminobenzoic acid (PABA) by yeast has been studied by Lampen, Baldwin & Peterson (169). Most of the PABA is in solution in the medium and very little is found in the yeast cells. Increases of twenty to eighty fold of PABA were obtained on synthetic media. No increase of PABA could be obtained after autolysis

of yeast and little evidence is presented to support the view that appreciable amounts of the vitamin occur in yeast in conjugated form.

Rubin and co-workers (170) have studied the responses of *S. cervisiae* and *L. arabinosis* to a biotin analogue (O-heterobiotin) which has the hetero-sulfur replaced by oxygen. While this analogue possessed some activity, it was not as active as *d*-biotin.

MISCELLANEOUS

A structural analogue of vitamin K (2,3-dichloro-1,4-naphthoquinone) possesses antifungal properties (171). It is harmful to yeasts and moderately harmful to bacteria. The presence of vitamin K, over a limited range of concentration, reverses the toxic effect of this analogue. The toxic effect on bacteria, however, is not counteracted by the presence of vitamin K.

Klein (172) believes that the *in vitro* virusidal activity of ascorbic acid can have no therapeutic applications because the presence of catalase in body tissues will destroy the hydrogen peroxide formed during the copper-catalyzed oxidation of ascorbic acid.

Benesch (173) determined nicotinic acid in human feces obtained from a cecostomy and cultivated aerobically and anaerobically. Synthesis of nicotinic acid occurred under aerobic conditions but cecal organisms destroyed 66 per cent of the nicotinic acid present under anaerobic conditions. The author suggests that in the normal cecum an equilibrium is struck between synthetic and destructive organisms. An upset in this equilibrium, he states, may play a part in the causation of deficiency diseases.

Examination of eleven different amides derived from pantothenic acid was reported with reference to possible antagonistic effects they might have on the bacterial growth-promoting properties of pantothenic acid (174). The most potent antivitamin activity was displayed by "pant-hydrazide."

Ryan *et al.* (175), in a study of the nutrition of *Clostridium septicum,* conclude that the organism is unable to synthesize the pantoyl moiety of pantothenic acid. They believe, however, that the organism can synthesize β-alanine and that this amino acid can be coupled with the pantoyl moiety of the medium to form pantothenic acid which is necessary for growth.

Snell & Shive (176) have studied the microbial growth-inhibiting effects of several chemical analogues of pantothenic acid. These ana-

logues consisted of pantothenyl alcohol and related compounds. All inhibited growth of some microorganisms which require pantothenic acid for growth. It is concluded that sensitivity of various microorganisms to "antimetabolites" varies widely. Similar results are also reported by the same workers (177) in a study with N-pantoyl-alkylamines and related compounds, as well as for α- and β-substituted analogues of pantothenic acid (178). Woolley & Collyer (179) report that the growth of all microorganisms studied was inhibited by phenylpantothenone.

Stansly & Schlosser (180) have advanced the hypothesis that the synthesis of pantothenic acid by *Escherichia coli* depends on the presence of pantoic acid or pantoate, rather than pantolactone as the precursor in pantothenic acid synthesis.

Natural and synthetic biotin are identical according to Stokes & Gunness (181). This conclusion was reached after studying the growth-promoting properties of five representative microorganisms. Both biotin preparations formed an inactive heat-dissociable complex with avidin.

Burkholder and co-workers (182) suggest that large-scale production of B_c concentrates may be commercially feasible by using selected strains of microorganisms. This is based on a study of autolyzed and enzyme-digested cultures of 369 yeasts, 82 strains of bacteria, and 84 molds, using *L. casei* to measure the folic acid concentration. A crude enzyme preparation from chicken liver was best for liberating the B_c factor from its conjugate. Similar observations by Daniel *et al.* (183) with reference to the chicken liver enzyme are recorded. Wright and co-workers (184) suggest that folic acid may undergo enzymic conversion in the liver of the rat with the formation of substances which have little or no activity as growth factors for microorganisms.

Choline analogues have been tested with cholineless mutants of *Neurospora* (185). The authors suggest that the gene-controlled deficiency in organism, "cholineless-2," may be concerned with the methylation of a mono- or dimethylated precursor of choline, while their "cholineless-1" organism blocks a prior step in choline synthesis.

Van Wagtendonk & Lamfrom (186) have reported further work on the "antistiffness factor" for guinea pigs. The distribution of acid-soluble phosphorus, in the muscles of guinea pigs afflicted with deficiency of the antistiffness factor, was deranged. Concentration of inorganic phosphorus increased during the deficiency, while creatine

phosphate and adenosinetriphosphate fluctuated and finally decreased. These workers are convinced that vitamin E and the antistiffness factor have different physiological functions in the animal organism.

Further attempts have been made to concentrate and purify the so-called B_{10} and B_{11} factors (187). Attempts to differentiate these factors from substances possessing high B_c (folic acid) activity have not been completely successful. The existence of a new hemoglobin maintenance factor is postulated.

Woolley & Sprince (188) have continued work on "unknown guinea pig factors" (189). They have now identified the unknown factor, "G.P.F.-1," as folic acid. Unknown factor "G.P.F.-2" is not a single factor. It is replaceable by a mixture of cellulose and casein or by a mixture of cellulose, arginine, cystine, and glycine. Factor "G.P.F.-3" has been concentrated but not identified.

Citrin is not identical with vitamin P, according to Scarborough (190) who believes that citrin is a crystalline complex of flavone derivatives which resemble a pure substance. It is stated that hesperidin and hesperetin, from orange meal, increased capillary resistance in man and rutin also gave positive results. Grapes are said to be the richest natural source of vitamin P.

NUTRITIONAL ASPECTS

Human Nutrition

Wilder (191) has recently published an excellent review in which he discusses the misinterpretations and misuses of the Recommended Dietary Allowances as issued by the National Research Council (192). The author defended the purpose and intent of the Food and Nutrition Board of the National Research Council in issuing their Recommended Dietary Allowances, in that the recommendations were based on the most reliable scientific knowledge available at the time and were also based on the requirements of normal people consuming daily diets containing from two thousand to three thousand Calories. In most instances the vitamin allowances have been optimal to the extent of allowing a margin of safety. The author acknowledges that the recommended allowances have not been found ideal for use with people of all ages, for people engaged in all types of work, or for people suffering from certain types of ailments. The author concludes his article by stating that the Recommended Dietary Allowances were

soon to be revised in the light of newer and more complete information. A few months after the Wilder article appeared, there was issued by the National Research Council a revised version (1945) of the Recommended Dietary Allowances (193) which differed from the 1943 issue primarily in that lower requirements for certain vitamins were indicated. It appears from these recommendations that lower requirements would have also been indicated for other vitamins had more complete information been at hand concerning the availability of these vitamins in human dietaries.

The National Research Council has sponsored a survey of the nutritional status of industrial workers (194). According to the report, all dietary studies indicate that a large portion of the industrial population is obtaining an unsatisfactory diet. While relatively few persons were reported as showing evidence of acute, severe nutritional deficiency diseases, there were signs and symptoms, often associated with proven malnutrition, exhibited by many people in all economic, occupational, and age groups. According to the report, malnutrition may result from limited income, poor food habits, ignorance, indifference, and the unavailability of proper foods. Poor food habits, ignorance, and indifference are the most important causes of malnutrition among industrial workers. The report goes further to state that mere knowledge of good nutrition does not make people give up bad eating habits or bad food practices. However, the most promising prospect of attaining better human nutrition seems to be through education.

In a study of the composition of large Navy messes in which as many as 7,500 men were fed daily, McCay et al. (195) found that the National Research Council's Recommended Dietary Allowances could be attained without difficulty, especially where liberal allowances of fruits and vegetables were permitted. Borsook (196) studied the effect of vitamin supplementation on absenteeism, labor turnover, and personnel ratings of aircraft workers in southern California. Data obtained under carefully controlled experimental conditions indicate that during the first six months of the test, both the control group of workers and the vitamin supplemented group had the same record of absenteeism. During the second six-month period, the control group had an average rate of absenteeism of 4.79 per cent while the vitamin supplemented group had an average rate of absenteeism of 3.90 per cent. The difference between the two groups of workers was considered statistically significant. According to the author, the group of

workers which had received the vitamin supplements not only lost less time from work, it also did more work and did it with less apparent effort than the control group.

Adamson *et al.* (197) made a study of the nutritional status of more than eight hundred people in the city of St. John's and the surrounding area. All individuals were examined clinically and chemical analyses were made of the body fluids of approximately half of the people comprising the group. The total number of analyses approximated four thousand. The food supply was also investigated. According to these investigators, evidences of deficiencies of vitamin A, riboflavin, and ascorbic acid were widespread. While mild, acute, and chronic niacin deficiencies were frequently observed, there was no evidence of rickets. In general, the diet was found to be adequate with respect to Calories, protein, and fat but extremely low in vitamin A, riboflavin, and ascorbic acid. There appeared to be a close parallelism between the food deficiencies and the clinical and chemical findings.

Johnson *et al.* (198) have reported on a series of field surveys of the nutritional status of native populations in special areas. The authors describe studies carried out by means of their field laboratory while operating in New England, Mojave Desert, Saskatchewan, and Colorado Rockies. The conclusions drawn by these investigators appear conservative in that they report, and even stress, that good health may exist under low storage of the various vitamins and that supplementing a normal ration with vitamins does not lead to a significant improvement in physical fitness. In another report (199) these authors point out in detail some of the precautions to be exercised while conducting vitamin status tests on soldiers.

Hrubetz *et al.* (200) studied the effect of high vitamin A intake on the vitamin A content of human milk. Experiments were carried out on forty-two women who received 50,000, 100,000, or 200,000 I.U. of vitamin A daily. The vitamin A content of milk from women receiving unsupplemented diets was found to vary with the period of lactation. For the first two to ten days, the milk contained an average of 331 I.U. per 100 ml., for the eleventh to thirteenth day, 232 I.U. per 100 ml., and for the thirty-first to sixtieth day, 171 I.U. per 100 ml. It is reported that 50,000 I.U. of vitamin A daily were found sufficient to produce an initial increase in the vitamin A content of human milk, that 100,000 I.U. daily were sufficient to maintain a constant vitamin A content, and that 200,000 I.U. daily resulted in the

production of human milk having three times the normal concentration of vitamin A.

The British Ministry of Foods initiated a study of the vitamin A requirement of humans in which conscientious objectors were used as the experimental subjects. The project was planned and supervised by the vitamin A Subcommittee of the Accessory Food Factors Committee, all members being considered authorities in the field of nutrition. After two years of study, during which the vitamin A requirement of twenty-three human subjects was investigated, a preliminary report has been issued (201). It seems to be the general conclusion that 2500 I.U. of vitamin A or 5000 I.U. of carotene daily may be regarded as adequate for maintenance of normal human adults, with a fair margin of safety. Details of the investigation are to be published at a later date. Anderson & Milam (202) have investigated the biomicroscopy of the eye as a means of evaluating human nutritional status. In a survey involving more than eleven hundred people, no correlation could be established between the severity of the incidence of conjunctivitis, as detected by means of the slit lamp, and the level of vitamin A in the diet or in the blood plasma. According to these investigators, conjunctival changes appeared more frequently and were of greater severity in the Negro than in white people.

Keys et al. (203) studied the effect of restricted intake of B vitamins on man. A rather comprehensive study is described using eight human subjects. Four of the subjects received diets adequately supplemented with B-complex vitamins, while the other four received B-complex deficient diets. Observations were recorded with respect to vitamin excretion, endurance, condition and fitness, intellectual function and response, hearing, strength, vision, muscular co-ordination, and cardiovascular capacity. The deficient group was characterized by the deterioration of endurance, co-ordination, and general fitness. Cardiovascular capacity and respiratory efficiency were also reduced. Changes in personality were manifested by apathy, depression, and hypochondriasis. Of the several vitamins under consideration, thiamine deficiency seemed to be more pronounced in these studies. Clinical methods used in determining vitamin requirements are described and discussed.

In an article summarizing his experience in human feeding, Keys (204) has discussed the human requirements for the B vitamins. This author emphasizes the need for human experiments as the means of arriving at vitamin requirements rather than those

based on animal experiments. He states that the human requirements for the B-vitamins are probably much lower than those indicated by the Recommended Dietary Allowances of the National Research Council and emphasizes the difference between "requirements" and "allowances."

Johnson *et al.* (205) studied the effect of ascorbic acid intake on the physical well-being of manual workers. Observations were made on four groups of laborers, each group receiving a different level of ascorbic acid intake and from 2400 to 5000 Calories daily. It was reported that, where the previous diet had been good, total deprivation of ascorbic acid for two months resulted in no detectable deterioration in physical vigor and efficiency of effort, provided the diet was adequate in other respects. A daily intake of 75 mg. of ascorbic acid restored the body level of ascorbic acid to normal in a relatively short period. Daily supplements of 75 mg. of ascorbic acid did not prove beneficial when given in connection with a good diet. As a result of information collected through a series of questionnaires, Ruskin (206) concludes that doses of ascorbic acid ranging from 250 to 750 mg. daily are definitely beneficial in allergies.

McHenry (207) has discussed the thiamine requirement of man from the standpoint of present-day allowances with reference to the fat content of the diet and its sparing effect on thiamine requirement. The author is of the opinion that the National Research Council "allowances" are too high, but does not suggest a more appropriate standard. In their study of the relative availability of thiamine in yeasts, Parsons *et al.* (79) fed different types of yeast to twenty-three human subjects and measured the elimination of thiamine in the urine and the feces. The data obtained as a result of these studies show that the thiamine of certain brands of yeast is very poorly utilized. In fact the data indicate that the presence of live yeast cells in the human digestive tract may interfere with normal thiamine metabolism. The thiamine of such yeasts was rendered available by boiling under specific conditions or by treatment with alcohol.

Alexander & Landwehr (208) studied the absorption of thiamine from the human colon by administering a mixture of thiamine and cocarboxylase by means of retention (24 hr.) enemas. Since there was no increase in urinary thiamine and since both thiamine and cocarboxylase were recovered as such from the twenty-four-hour stool, the authors conclude that any thiamine which may be formed in the human colon is not available.

CLINICAL

Krestin (209) studied the antirachitic effect of massive doses of vitamin D_2 on rachitic children. In this study, eighty-three children under two years of age were studied radiologically following doses of vitamin D_2 ranging from 300,000 to 600,000 I.U. Because of the favorable response, the author advocates this method of vitamin D therapy since no toxic effects were noted and since it represents an economical procedure. On the other hand, Donowski et al. (210) reported tissue calcification and renal failure produced in two arthritic patients by massive doses of vitamin D.

Siddall & Mull (211) studied human urinary thiamine excretion in forty-two women as affected by stage of pregnancy. They found that while urinary thiamine generally decreased with increase in prenatal development, there were other factors such as vomiting, poor appetite, or multiple pregnancy which also affected urinary thiamine output.

Of some nine hundred pregnant women examined in Palestine, Braun et al. (212) observed that one hundred and ninety suffered from glossitis and heartburn, often accompanied by cheilosis, angular stromatitis, and corneal vascularization. The symptoms were usually observed during the last trimester of pregnancy. That these conditions were due to riboflavin deficiency was indicated by low urinary riboflavin and by the response of the patients to riboflavin therapy. In their studies on corneal vascularization, Anderson & Milam (213) could establish no correlation between corneal invasion and dietary riboflavin. These authors report that corneal invasions are observed more frequently among white people than among Negroes. Cayer, Ruffin & Perlzweig (214) have also reported observations which seem to support the contention that cheilitis is nonspecific and is not usually caused by a riboflavin deficiency. Spies et al. (215), in the light of their extensive experience with ocular disturbances associated with riboflavin deficiencies, have published an excellent review of the early literature and the results of clinical examinations of thousands of people, with a description of techniques and clinical tests and with typical case descriptions. The specific cases of three hundred individuals are submitted to substantiate the contention that the nutritional status of all patients with ocular lesions or cheilosis be carefully studied and that riboflavin be administered as a therapeutic agent.

Cayer et al. (216) observed that multiple B-complex deficiencies

are regularly observed in patients afflicted with sprue and that plasma levels of vitamin A and carotene are also significantly subnormal in these patients.

Briggs et al. (217) studied the effect of niacin destruction in man. Two subjects, previously suffering from pellagra, were restricted to a diet low in trigonelline and which furnished only 3 mg. of niacin daily for periods of thirty-six and forty-two weeks, respectively. Although niacin and trigonelline elimination remained low throughout the test, the pellagrous condition did not become worse. The authors suggest that the failure of these patients to develop pellagra under the conditions of the experiment may have been due to intestinal synthesis or to lack of corn in the diet. Sevringhaus et al. (218) observed that 50 mg. doses of nicotinamide caused improvement in unselected prisoners afflicted with lingual atrophic changes. Field et al. (219) reported that six cases of glossitis and one case of cheilosis responded to treatment with calcium pantothenate when treatment with other vitamins had failed. Rose, Duane & Fischel (220) reported a case of Rocky Mountain Spotted Fever which received significant benefit from treatment with *p*-aminobenzoic acid.

Berry et al. (221) reported observations which suggest that in some instances the use of an intravenous liver-concentrate containing folic acid elevated the total number of circulating leucocytes with a proportional increase in granulocytes for varying periods. The *L. casei* factor caused slight but temporary rise in the leucocyte count.

Davidson (222) has stated that the use of B-complex vitamins is indicated in septic surgical practice, especially in cases of prolonged or extensive bleeding.

Gaebler et al. (223), in their experiments with dogs afflicted with experimental pancreatic diabetes, observed that when yeast or a mixture of vitamins consisting of thiamine, riboflavin, niacin, inositol, pyridoxine, pantothenic acid, and *p*-aminobenzoic acid were added to the diet of the diabetic dog, there was a measurable decrease in insulin requirement.

ANIMAL NUTRITION

The importance of vitamins in the nutrition of domesticated animals is attested by the fact that the National Research Council has issued recommended nutrient allowances for poultry (224), swine (225), and dairy cattle (226), each of which include suggested vitamin allowances.

Sherman & Campbell (227) studied the stabilizing influence of varying intakes of vitamin A on the rate of growth of young rats. Three levels of vitamin intake were investigated. Rats which received the higher levels of vitamin intake grew at a more rapid rate and with less growth variations than those on the lower vitamin intake. In a continuation of these studies (228) the authors found that adequate vitamin A intake in rats resulted in an extended reproductive period and a greater length of life. Moore & Wang (229) produced hypervitaminosis-A in rats by feeding massive doses of crystalline vitamin A acetate and halibut liver oil. The most characteristic lesions observed were fragility of bones and internal and external hemorrhages. Cross (230) investigated the relationship between the weight of young vitamin A-deficient rats at the end of depletion period and the subsequent growth response made when β-carotene was administered, but failed to find that any definite relationship existed. Fraps & Meinke (231), by measuring the carotene intake of rats and the carotene excreted in the feces, estimated the relative digestibility of the α-, β-, and neo-β-carotene content of a number of vegetables. Digestibility data are presented and discussed by these authors.

Kemmerer et al. (232) studied the relative value of carotene in different vegetables by comparing their capacity to promote growth in rats. Using carotene in oil as their standard, they reported that twenty-seven vegetables and feeds contained carotene of high biological value. Deuel et al. (233), using rats as their test animals, reinvestigated the relative provitamin A potencies of cryptoxanthin and β-carotene. The results obtained in two independent bioassays indicate an actual provitamin ratio of 54 : 100 and 59 : 100. Dye, Bateman & Porter (234) studied the effect of the level of protein intake on the utilization of vitamin A by the rat and was unable to detect any definite relationship.

Sutton et al. (235) investigated the carotene and vitamin A content of blood plasma of dairy cows with reference to variations associated with parturition and lactation. These investigators found that plasma carotene decreased markedly at the time of parturition and during the first week of lactation. The minimum plasma vitamin A was observed about the third day after parturition. Large reserves of carotene and vitamin A were eliminated in the colostrum during the first three days following parturition.

Braun (236) studied the seasonal changes in the carotenoid and vitamin A levels and the normal carotenoid-vitamin A ratio in bovine

blood. Seasonal changes in blood carotene levels were found to depend primarily on the diet of the cow but they also varied according to age and breed. Seasonal changes in blood vitamin A were influenced primarily by the carotene and the vitamin A intake. Vitamin A in the blood was found to decrease with parturition, abortion, and acute infection. This author reports that a linear relationship exists between the carotene-vitamin A ratio in bovine blood when the diet is rich in carotene. In a subsequent investigation, Braun (237) studied carotene and vitamin A storage in the liver of cows from four different dietary groups. The results of these studies show significant differences in vitamin A storage in three out of four of the experimental groups. Analyses of livers from depleted cows indicate that carotene disappears from the liver before the vitamin A level is seriously affected. The author states that the blood of the cow tends to maintain a definite carotene-vitamin A ratio on normal diets. Sutton & Soldner (238) investigated the seasonal variations in the vitamin A and the carotene content of blood from adult dairy cattle at monthly intervals over a two-year period. Representative animals from four dairy breeds were used. Blood carotene values were found to vary more extensively than blood vitamin A values.

Moore & Berry (239) investigated the vitamin A and carotene content of blood plasma of three breeds of dairy calves from birth to four months of age. The data are presented and discussed. Lewis & Wilson (240) investigated the vitamin A requirement of calves and reported the minimum requirement as being 32 I.U. per kg. of body weight. For optimal growth and normal liver storage, these authors recommend 250 I.U. per kg. of body weight. Shaw et al. (241) investigated the possible relationship between ketosis in dairy cattle and vitamin A deficiency. Blood tests on eight animals afflicted with ketosis showed blood carotene and blood vitamin A to be normal. Oral administration of four million I.U. of vitamin A had no beneficial effect but the condition was cured by pasturing on green grass. The authors conclude that ketosis in dairy cattle is not caused by vitamin A deficiency.

Brown & Bloor (242) investigated the nutritive value of the fatty acids of butter and their effect on the utilization of carotene. The fatty acids of butter were separated into five fractions, each of which was employed to replace milk fat in a normal diet for rats. The liquid fractions appeared to stimulate carotene utilization and vitamin A storage.

Wheeler (243) studied the effect of high vitamin A diets on domesticated and nondomesticated animals. She reported that when high vitamin A diets were fed to dogs and rabbits a persistent diarrhea resulted due to over-stimulated metabolism.

Bentley & Morgan (244) studied the vitamin A and carotene nutrition of the guinea pig and found that this animal manifested essentially the same deficiency symptoms as other species. Small stores of liver-vitamin A were observed when carotene was fed to the guinea pig but when vitamin A was fed large liver storage resulted.

Bohren, Thompson & Carrick (245) studied the transfer of carotenoid pigments from the diet of the hen to the egg yolk by feeding high and low carotenoid-containing diets and by analyzing the eggs for total carotenoids at specified intervals. These authors report that egg yolks from hens receiving carotenoid-rich rations decline rapidly in pigment content during the first ten days after the hens are placed on a pigment-free ration, after which the decline in yolk-pigment is more gradual. After eighty-four days on the pigment-poor ration the hens produced eggs the yolks of which still contained two or three times as much pigment as egg yolks from hens which had always received the pigment-poor rations.

Stott & Harris (246) have suggested a synergistic effect of cod liver oil on synthetic vitamin D_3, but this view is not shared by Graves (247).

Singsen & Mitchell (248) have studied the relationship between phytin and different sources of vitamin D. According to these authors the vitamin D from irradiated animal sterols is 1.34 times as effective as those from cod liver oil in promoting calcification in chicks which depend on phytin as the principal source of phosphorus.

In a search for a more desirable test animal for use in rachitogenic studies, Jones (249) found that the hamster, like the rat, requires an adverse calcium-phosphorus ratio for the production of satisfactory experimental rickets.

Willman et al. (250) investigated the relationship of vitamin E to "stiff-lamb disease" and found that by feeding mixed tocopherols to ewes and lambs or by injecting dl-α-tocopherol phosphoric acid ester, the stiffness could be prevented.

Patrick & Morgan (251) studied the role of vitamin E in chick nutrition and conclude from their results that the chick requires this vitamin for optimal vitamin A utilization.

Lecoq et al. (252) studied the storage of ascorbic acid in various

tissues of guinea pigs subsisting on scorbutogenic diets with and without various supplements. It was found that the guinea pig, unlike the rat, was unable to store appreciable amounts of ascorbic acid in its tissues unless the diet was rich in this substance. Calciferol appeared to exert a depressing effect on ascorbic acid storage.

Parsons *et al.* (253) studied the availability of thiamine in various types of bakers' yeast for growth in rats and found that the thiamine was relatively unavailable in six of the eight yeasts investigated. Two samples of yeast which produced the greatest biological response also contained the highest thiamine content. According to these authors the thiamine in fresh yeast became available more readily when the yeast was boiled or when the yeast was treated with alcohol. Kline, Friedman & Nelson (254) studied the effect of environmental temperatures on the thiamine requirement of the rat. Using the curative test as the criterion of availability, the authors conclude that the rat requires less thiamine at higher temperatures than at lower temperatures. The decrease in thiamine requirement was reported to parallel the decrease in caloric requirement. The above conclusions were essentially substantiated by the findings of Edison *et al.* (255).

Richter & Rice (256) and Richter (257) studied the relationship between dextrose and sucrose intake on thiamine utilization by the rat. Using the survival time as an index of availability, the authors found no significant difference between the two carbohydrates so far as thiamine utilization was concerned.

Waisman *et al.* (258) studied the effect of fat and sodium pyruvate on the susceptibility of thiamine deficient mice to poliomyelitis virus. This was an attempt to confirm the hypothesis that keto acids, formed during thiamine deficiency and accumulated during abnormal carbohydrate breakdown, interfere with the invasion of the poliomyelitis virus. The experiments were not completely successful. Further studies reported from the same laboratory (259) in which monkeys were used as the test subjects show that this animal is no more susceptible to poliomyelitis when suffering from thiamine deficiency than when receiving a normal diet.

Weaver *et al.* (260) investigated the resistance of the cotton rat to the virus of poliomyelitis as affected by intake of the B-vitamins, by partial inanition, and by sex. The results of the experiments show that deficiency of the B-complex, or partial inanition, or sex has no significant effect on susceptibility to the infection. Thus the results of the study do not support the theory that the virus of poliomyelitis may

be a biochemical by-product of thiamine deficiency or that poliomyelitis is a fulminant form of beri-beri.

The studies of Pence *et al.* (261) indicate that, with high vitamin intake, the thiamine is rapidly stored in pork muscle, the tissue becoming saturated in thirty-five days or less. However, when the pig is changed from a thiamine-rich to a thiamine-poor diet, pork muscle loses its thiamine very slowly.

As a result of their studies on rats, Lowry & Bessey (262) conclude that the variety of agencies and deficiencies which will induce corneal vascularization makes it imperative that caution should be exercised in interpreting corneal vascularization in man. In their studies relative to the nutritional requirements of the monkey, Cooperman *et al.* (263) found that riboflavin deficiency in the rhesus monkey was characterized by cessation of growth, anemia, and a dermatitis. With this animal, saturation tests failed when applied as the diagnostic means of determining riboflavin requirement. The minimum riboflavin requirement was found to be about 25 to 30 μg. per kg. of body weight. An unknown factor in whole liver was found to be necessary for optimum growth and for blood regeneration in the monkey.

As the result of examining horses afflicted with periodic ophthalmia, Jones *et al.* (264) conclude that the lesions possess striking similarity to those caused by riboflavin deficiency in experimental animals. Ocular fluids from normal horses were found to be high in ascorbic acid while horses afflicted with periodic ophthalmia were found to have a low concentration of ascorbic acid in the ocular fluid. These authors advance the hypothesis that the lack of dietary essentials, particularly riboflavin, influences the synthesis of ocular ascorbic acid or its protecting substances.

Villela & Prado (265), in their investigation of the vitamin content of the blood plasma of some Brazilian snakes, found that the plasma from two species of snakes contained an average of 200 μg. of riboflavin per 100 ml. Xanthophyll was found to be present in snake plasma but carotene was absent.

Wooley & Sebrell (266), in their studies on the nutritional requirements of the rabbit, found that in order to produce growth in this species, niacin was an essential supplement to a purified ration. Wintrobe *et al.* (267) found that high protein intakes appeared to have a sparing effect on the niacin requirement of the pig.

Teply, Krehl & Elvehjem (268) investigated the biological activity of N-methylnicotinamide and nipecotic acid (hydrogenated

nicotinic acid). The latter compound was found to be as effective as niacin in producing growth in *L. arabinosus,* while the chloride of the former compound failed to cure black tongue in dogs. The latter observation is contrary to the findings of Najjar *et al.* (269). Krehl, Teply & Elvehjem (270) investigated corn as an etiological factor in the production of niacin deficiency in the rat and found this cereal to be unique in its growth-depressing effect on rats subsisting on low niacin diets. This surprising effect of corn does not appear to be directly related to its low niacin content. In a later publication from the same laboratory, Krehl *et al.* (271) reported that tryptophane counteracted the growth-retarding effect of corn in niacin-low rations. These authors report that 50 mg. of *l*-tryptophane or 1.0 mg. of niacin per 100 gm. of ration completely counteracted the growth retardation caused by including 40 per cent of corn grits in a low protein ration. In further studies Krehl *et al.* (272) found that when corn grits replaced 60 per cent of the sucrose in the diet of the dog, an increase in niacin requirement resulted. They attribute the increased niacin requirement to a decreased synthesis of the vitamin in the digestive tract when the corn diet is consumed.

Richards (273) has reported that pyridoxine deficiency in the rat can be accentuated by overloading the diet with thiamine. Miller & Baumann (274), using their own method for estimating xanthurenic acid in urine, found that pyridoxine-deficient mice excreted this chromogen in amounts depending upon the casein or the tryptophane content of the diet. Results with rats were similar but were not as clear-cut as those obtained with mice. Axelrod *et al.* (275) have reported additional evidence that tryptophane metabolism is disturbed in pyridoxine deficiency. Pyridoxine-deficient dogs were found to convert tryptophane to xanthurenic acid which is excreted as such in the urine.

Hegsted & Rao (276) observed that acute pyridoxine deficiency in young ducklings is manifested by cessation of growth and anemia while chronic pyridoxine deficiency in older ducks is evidenced by paralysis, convulsion, microcytic anemia, and poor feather development.

Seeler & Silber (277) investigated the pantothenic acid requirement of dogs and found that some dogs lived for more than four years on a purified basal diet complete in all known required nutrients except pantothenic acid. Pearson *et al.* (278) compared the pantothenic acid content of eggs with the amount of this substance found in the

newly hatched chick. It was found that during embryonic development of the chick, pantothenic acid tends to remain at a constant level, regardless of the pantothenic acid content of the egg at the beginning of incubation. The amount of pantothenic acid in the egg and in the newly hatched chick was found to be related to the pantothenic acid content of the hen's diet.

Lepkovsky and co-workers (279) have reported that chicks require from 4.6 to 9.1 mg. of pantothenic acid per kg. body weight for normal growth, while turkey poults require from 9.7 to 11.7 mg. of the vitamin for growth while subsisting on similar rations. The requirement of chicks and poults for pantothenic acid was found to be influenced by their relative rates of growth, the faster growing birds requiring the greatest amount of the vitamin.

Victor et al. (280) observed that 1 per cent of dietary choline had an inhibiting effect on the deposition of liver ceroid resulting from the ingestion of low protein diets containing an excess of cystine. Under similar conditions α-tocopherol exhibited a transient inhibitory effect. McKibbin and co-workers (281) found that when choline was added to the choline-deficient ration of pups there resulted a rapid increase in food consumption and in body weight, an improvement in liver function, and a withdrawal of lipids from the liver. Normal liver function was restored in from five to ten days. Kesten et al. (282) observed that the feeding of ethyl laurate at 35 to 40 per cent levels in the diet of young choline-deficient rats resulted in death of the test animals in three to six days, due to heart failure. The pathological picture was that of acute diffused interstitial myocarditis. According to these authors this disease did not develop when other ethyl esters were fed and was not fatal when the concentration of ethyl laurate was reduced to 25 per cent of the diet.

Supplee et al. (283) have determined the "choline equivalent" of casein, lactalbumin, and soybean protein. The amount of protein which is equal to choline in transmethylation ability was determined by preventing liver and kidney damage in rats. It was found that casein was superior to other proteins tested in preventing the characteristic fatty deposition in the liver.

Through the use of diets deficient in methionine and cystine, Almquist & Grau (284) conducted feeding experiments with chicks in which the level of cystine was increased gradually to the optimal sulfur amino acid content, with the addition of various levels of choline. The authors conclude that cystine apparently cannot com-

pensate for a methionine deficiency, even when high levels of choline are employed.

In their studies on the interrelation of fats, carbohydrates, and B-vitamins in rat nutrition, Boutwell and co-workers (285) found that different types of fats altered the apparent vitamin requirement of the rat. Better growth was produced by diets containing butterfat than by similar diets containing corn oil. Apparently the corn oil prevented the intestinal synthesis of certain B-vitamins to a greater extent than did butterfat. When lactose was fed as the carbohydrate or when the diets contained adequate amounts of the B-vitamins, butterfat did not prove superior to corn oil in promoting growth. Jones *et al.* (286) produced thiamine, riboflavin, pyridoxine, and pantothenic acid deficiencies in mice and described the most characteristic deficiency syndrome in each case.

Lichstein and co-workers (287) studied the effect of pyridoxine, inositol, and biotin on the susceptibility of Swiss mice to experimental poliomyelitis. Pyridoxine, inositol, and biotin, when present or absent from the diet, failed to influence the susceptibility of Swiss mice to the Lansing strain of poliomyelitis. In their studies regarding the monkey anti-anemia factor, Cooperman *et al.* (288) found that fresh liver was more potent than whole liver powder in the anti-anemia factor. Beef and pork liver were found to have equal potency and lyophilized liver retained all the active principle of fresh liver.

Shaw & Phillips (289) produced pantothenic acid, biotin, and folic acid deficiencies in growing chicks and studied the degenerative changes in certain nerve tissues by means of the polarizing microscope. In pantothenic acid deficiency they found myelin and axon degeneration in the spinal cord. No characteristic degenerative changes in nerve tissue could be associated with biotin and folic acid deficiencies.

Richardson, Hogan & Kempster (290) have reported that when a practical poultry ration was fed to day-old chicks, the ration was found to be deficient in pantothenic acid and choline, slightly deficient in niacin and biotin, and deficient in at least one unrecognized dietary essential.

Miller (291) observed that when highly purified diets containing 0.5 to 2.0 per cent of succinylsulfathiazole or phthalylsulfathiazole were fed to rats over a long period, nutritional deficiency symptoms developed which were cured by feeding biotin and folic acid. The intestinal flora changed considerably and less biotin, folic acid, and pantothenic acid were excreted in the feces than with the same diets

without the sulfa drugs. Lindegren & Lindegren (292) have shown
that the inability of yeast to synthesize certain vitamins may be over-
come by hybridization and that the heterozygote usually synthesizes
the vitamins about as well as the homozygote. Vinson *et al.* (293)
have reported that *Fusarium lini* B contains enough of the B-vitamins
to meet the nutritive requirement of the mouse when grown on a
medium containing adequate amounts of these vitamins. When
grown on this type of medium, *Fusarium lini* B compares favorably
with yeast in nutritive value.

<center>MISCELLANEOUS</center>

Snell (294) has published an excellent resumé of the available
information relating to the nutritional requirements of lactic acid bac-
teria and their biochemical applications. In their studies of spotted
fever, Anigstein & Bader (295) observed that *p*-aminobenzoic acid
rendered guinea pigs almost immune to infection when subsisting on
high protein diets. Control animals which did not receive the *p*-amino-
benzoic acid died within five to eight days. *p*-Aminobenzoic acid did
not always prevent some of the febril symptoms but did preserve life
when given twenty-four hours after the pigs had been infected.

Using incubated hen's eggs and injecting suspensions of the rickett-
siae in the presence and absence of *p*-aminobenzoic acid, Hamilton
(296) observed that maximum doses of the vitamin were effective in
inhibiting rickettsiae of spotted fever. The inhibiting effect of PABA
was expressed in the following order of magnitude: Rocky Mountain
s.f.>Brazilian s.f.>Colombian s.f.>South African Tick f. *p*-Amino-
benzoic acid was found to be about twice as effective against the Rocky
Mountain spotted fever infection as against the epidemic or Murine
typhus type. The author suggests that such a high degree of specificity
indicates that *p*-aminobenzoic acid may function in an enzyme system.

Snyder *et al.* (297) found that the mortality of rodents, which have
been injected or fed an inoculum of *R. orientalis,* could be decreased
by feeding diets containing *p*-aminobenzoic acid. This confirms the
findings of Hamilton. Martin & Rose (298) studied the antibacterial
activity of thirty-five substances closely related to *p*-aminobenzoic
acid. Three of the compounds, 3-hydroxy, 3-chloro-4-amino, and
3,4-diaminobenzoic acid, were found to be inhibitory.

Hickey (299) has reported that the inactivation of iron by means
of 2,2-bipyridine in the fermentation of corn mash by *Cl. acetobutyli-
cum* resulted in an increase in riboflavin production. However, when

molasses was used as the fermentation medium, no increase in riboflavin resulted.

In their studies relative to the nutritional requirements of various insects, Fraenkel & Blewett (300) found that only those insects belonging to the genus *Ephestis* required linoleic acid, vitamin E, or other fat-soluble factors contained in wheat germ.

METHODS OF ASSAY

BIOLOGICAL METHODS

Fat-soluble vitamins.—Fraps & Meinke (301) studied the relative biological values of the carotene of six different foods by determining the amount of the vitamin stored in the livers and excreted in the feces as a result of feeding 60 μg. of carotene daily to rats over a fourteen-day test period. Using carotene in oil as their vitamin standard, these authors found that carotene as it exists in vegetables possessed relatively low biological value while carotene in butter and in beef liver possessed high potency. Callison & Orent-Keiles (302) have studied vitamin A standards most frequently employed in biological assays. They concluded that the U.S.P. reference cod liver oil (No. 2) contains less than 1700 I.U. per gm. These authors recommend the use of pure β-carotene in place of the U.S.P. reference cod liver oil as the standard.

Jones (303) carried out a rather critical study of the chick assay method for estimating vitamin D, with the view of completely eliminating or of reducing the magnitude of some of the variations frequently experienced when the method is employed. In these studies, the calcium and phosphorus content of the diet received special emphasis. The author found that maximum reliability was obtained with the bone-ash method when the chick diet contained calcium and phosphorus in the ratio of 1.5 per cent calcium to 1.5 per cent phosphorus. Campbell and co-workers (304 to 307) investigated the chick methods of vitamin D assay with respect to the most reliable criteria of recalcification. According to these investigators, the toe-ash method of assay offered definite advantages. In a careful statistical study of the comparative accuracy of data obtained for vitamin D by both tibia-ash and by toe-ash methods, Bliss (308) confirms reports from other laboratories that vitamin D assays can be made more easily and just as accurately by the toe-ash method, rather than to use the more time-consuming official A.O.A.C. tibia-ash method.

Water-soluble vitamins.—Obermeyer & Chen (309) estimated the biological value of thiazole and pyrimidine moieties of thiamine by measuring the capacity of bakers' yeast to synthesize thiamine in the presence of these substances. Differential assay procedures were said to have permitted the estimation of thiamine, thiazole, and pyrimidine without interference from other substances. These investigators concluded that substantial amounts of thiazole and pyrimidine moieties may occur in food products and that the amounts vary with different foods. Melnick, Hochberg & Oser (310) and Oser, Melnick & Hochberg (311) describe a biological assay technique, using human subjects, in which the physiological availability of water-soluble vitamins is determined. The assay is based on the assumption that water-soluble vitamins, as such or as their derivatives, are excreted in amounts paralleling the quantities consumed. These investigators claim a linear relationship in the dose-response for the vitamins under test and that the method of assay yields data which are more readily reproduceable than data obtained by the usual animal assay methods. This method of assay is believed to be useful in determining the biological availability of the water-soluble vitamins as they exist in mixed diets or in multiple vitamin preparations.

CHEMICAL METHODS

Fat-soluble vitamins.—Since certain vegetables, especially carrots, constituted an important source of provitamin A in England during the war period, it seemed desirable that a rapid method of assay should be developed for the estimation of the carotene content of carrots. Booth (312) has described a simplified method for the estimation of the carotenoid content of this vegetable. The method involves the extraction of the plant pigments with a petroleum ether-acetone mixture and the estimation of the carotene content by colorimetry. About 90 per cent of the total carotenoids were estimated to be carotene.

Caldwell & Parrish (313) found that light of high intensity caused rapid fading of the blue color formed by the Carr-Price reaction used in estimating vitamin A. In order to reduce fading to a minimum and thereby increase the accuracy of the method, the author recommends the employment of lights of low intensities.

Sobel, Mayer & Kramer (314) made spectrophotometric studies of colored compounds formed when vitamins D_2 and D_3 and their provitamins reacted with glycerol trichlorohydrin and acetyl chloride. By this procedure, the authors claim it is possible to differentiate

vitamins D_2 and D_3 from ergosterol and 7-dehydrocholesterol; ergosterol from 7-dehydrocholesterol and the dinitrobenzoate esters of the above compounds. Thibaudet (315) reported studies in which calciferol was separated from tachysterol by chromatographic means and measured quantitatively by a modified Carr-Price procedure.

Kofler (316) determined the tocopherol content of plant and animal tissues. Using a fluorometric method, this author found that fresh plant and animal tissues do not contain tocopherol quinone, while dried plant tissue may contain up to 50 per cent of the total tocopherol in this form. Young animal tissues were found to be lower in tocopherols than comparable tissues from older animals. Beef brain was found to be richer in this substance than human brain. Chipault, Lundberg & Burr (317) employing a chemical method, determined the tocopherol content of various animal fats. According to these authors, fats from different parts of the hog's body contain similar amounts of tocopherols, while fats from different hogs often vary widely in tocopherol content. As a result, the authors conclude that the tocopherols are the only natural oxidation inhibitors in hog fat and that the tocopherols of hog fat are probably derived solely from dietary sources. Fisher (318) has proposed a method for estimating the γ-tocopherol content of vegetable oil in the presence of α-tocopherol but in the absence of β-tocopherol. The method consists essentially of oxidizing the tocopherol with nitric acid in the presence of acetic acid and measuring the resulting red color by photometric means.

Owing to loss of water-soluble solids during blanching, Lee (319) has concluded that the carotene content of blanched carrots, when expressed on the basis of alcohol-insoluble solids, is a more reliable criterion of vitamin retention than total dry matter.

Vavich, Stern & Guerrant (320) found that a final concentration of 3 per cent of metaphosphoric acid was necessary to stabilize the ascorbic acid in fresh unblanched peas, during maceration in a Waring blendor. An atmosphere of nitrogen was found to favor the conservation of ascorbic acid in peas, especially where the metaphosphoric acid concentration was less than 3 per cent. Tuba, Cantor & Hunter (321) assayed rose hips for ascorbic acid by the conventional dye-titration method and by the biological method of assay, using the guinea pig growth procedure and the serum phosphatase procedure. The last mentioned assay procedures yielded data which agreed with those obtained by the dye-titration method. The authors conclude that the dye-titration method affords an accurate measure of the ascorbic acid

content of dried rose hips. Nelson & Somers (322) have made use of a modification of the indophenol-xylene extraction method in estimating the ascorbic acid content of a relatively large number of samples of tomatoes and tomato juices. On the other hand, Stewart & Sharp (323) have attempted to develop a method for assaying ascorbic acid in the presence of reducing substances which interfere with the conventional method of assay. The method which they propose is based on a selective oxidation-reduction procedure using ascorbic acid oxidase (from cucumbers) as the oxidizing agent and by reducing the dehydroascorbic acid with *Escherichia coli* or with *Staphylococcus albus*. The reduced ascorbic acid is then determined by the usual dye-titration procedure. The method is said to have been applied successfully in determining the ascorbic acid content of milk, milk products, fruit and vegetable juices, urine, and blood plasma. Likewise, Robinson & Stotz (324) have proposed modifications of the indophenol-xylene extraction method for estimating ascorbic acid which are said to minimize or eliminate the errors due to interfering substances. These authors submit data to show that errors due to reductones and reductic acid are largely eliminated. No means were found by which the interfering effects of sulfhydryls and quinol compounds could be eliminated. Lowry and co-workers (325) have reported a micro-method for use in estimating the ascorbic acid content of small amounts of blood serum. The procedure is essentially an adaptation of the dinitrophenylhydrazine method of Roe & Kuether and is said to be applicable to as little as 0.01 ml. of blood serum. The proposed method seems to offer definite advantages when used in nutritional survey studies where large numbers of samples are involved.

Hochberg and co-workers (326) have reported what they describe as a simplified colorimetric method which they found applicable for the determination of thiamine in cereals. The method consists of the preparation of a clear extract of the cereal, adsorbing the vitamin on zeolite and eluting, reacting with diazotized *p*-aminoacetophenone, extraction with xylene, and measuring the color. The method is said to be simple and inexpensive. By carefully controlling the pH in the final stages of extraction, Mickelsen and co-workers (327) believe they have eliminated the major source of errors encountered in determining urinary thiamine by the thiachrome method. The interfering fluorescent substances are eliminated and the urinary thiamine values are found to be somewhat higher for thiamine-deficient patients. The data presented are treated statistically. Perlzweig and co-workers

(328) have likewise suggested certain modifications of existing thiamine assay methods with the view of adapting a method for the determination of thiamine in urine. In order to have available at all times uniform thiamine and riboflavin standards, Stamberg & Bolin (329) have suggested that the standards be prepared in 2 per cent acetic acid solution, placed in special vials, and maintained in a frozen state until needed.

Johnson and co-workers (330) have reported a rapid analytical procedure which they used in field studies in assaying thiamine, riboflavin, and methylnicotinamide. Data are submitted to show the reproduceability of the methods. Using enzyme-digestion previous to adsorption, Rosner, Lerner & Cannon (331) found that higher riboflavin values were obtained. They explain the higher values on the basis of a breakdown of the riboflavin complex, thereby rendering the free vitamin adsorbable. These observations appear to be in agreement with those previously reported by Rubin *et al.* (332, 333).

In a study of the Konig reaction, Friedemann & Frazier (334) observed that nicotinic acid, on reacting with cyanogen bromide and certain substituted aromatic amines, yielded glutaconic dialdehyde derivatives which fluoresced with a greenish-yellow hue when exposed to visible violet light of about 440 mμ. This fluorescence was evident when the solution contained as little as 0.02 μg. of niacin per ml. The authors suggest that the principle of preparing fluorescing "tagged" derivatives be applied to other vitamins of the B-complex. In their studies relative to urinary excretion of N^1-methylnicotinamide, Hochberg, Melnick & Oser (335) employed an analytical procedure which involved the adsorption of this metabolite on activated zeolite, elution with a potassium chloride solution, treatment of the eluate with alkali, and a comparison of the butanol solution with standards, similarly prepared, by means of a fluorometer.

Brown, Bina & Thomas (336) have reported a method which they used in assaying pyridoxine which was based on a diazo reaction using diazotized *p*-aminoacetophenone. A synthetic resin "Amberlite IR-4" was used to remove interfering substances and alkaline ethyl alcohol was used as the eluting agent. Good recoveries were reported. Entenman & Chaikoff (337) have described analytical procedures which they employed in determining the choline content of the liver and plasma of the dog. They found that approximately 50 per cent of the plasma choline was in the free form.

Guerrant, Vavich & Fardig (338) made a study of the results ob-

tained by applying some of the accepted methods of vitamin assay to samples of six specially prepared canned foods. Samples of the same products were assayed by collaborating laboratories. The results were sufficiently variable to lead to the conclusion that there is definite need for extensive collaborative studies in order to eliminate inconsistencies existing between methods, materials, and details of procedures used by various investigators.

PHYSICAL METHODS

Fat-soluble vitamins.—Wilkie & De Witt (339) have described a direct spectrophotometric method for estimating the vitamin A content of oleomargarine. The method involves saponification and chromatographing the nonsaponifiable residue. Precautions are described for preventing or minimizing the destruction of the vitamin during the assay. Sobel & Werbin (340) describe a method for estimating vitamin A which involves a new reagent, 1,3-dichlorohydrin instead of antimony trichloride. The authors claim that when the dichlorohydrin is used the resulting coloration is stable for from two to ten minutes, thus permitting ample time for adsorption measurements. Kascher & Baxter (341) investigated a large number of fish liver oils with respect to their vitamin A content. The method was based upon phasic separation between petroleum ether and 83 per cent aqueous ethanol, followed by quantitative molecular distillation. It was concluded that 95 per cent or more of the vitamin A in fish liver oils is present in the ester form. Little or no free vitamin A could be found. The authors state that methods based on fluorescence were found to give erroneous results. Oser *et al.* (342) carried out a rather comprehensive study of vitamin A assay methods in which more than a hundred vitamin A-containing substances were assayed by spectrographic, colorimetric, and by multiple-level bioassay methods. According to these authors results of these studies caused considerable doubt regarding the reliability of the data obtained by the spectrographic method of assay, while the special colorimetric procedure developed by the authors yielded data which showed good agreement with those obtained by the bioassay method.

Cooley and co-workers (343) have reported a method for the estimation of the vitamin A content of mixed feeds. In their procedure the crude extract from the feed is chromatographed by passing through a column containing finely pulverized sodium carbonate

which adsorbs interfering substances but permits vitamin A and carotene to pass through. Excellent recoveries are reported. Metcalf (344) has suggested that vitamin technologists express the vitamin content of fish liver oil in terms of micrograms of vitamin per gram of oil rather than as International Units per gram. This author also urges that the $E_{1cm.}^{1\%}$ 328 mμ be stated for each oil assayed and that a factor be established for converting "E" values to micrograms of vitamin per gram of oil. It is believed that these steps would do much toward eliminating a major portion of the trouble now experienced by commercial analysts in expressing the vitamin A potency of fish oil.

Water-soluble vitamins.—Gillam (345) has reported a polarographic method for estimating ascorbic acid which is said to yield data in excellent agreement with those obtained by the dye-titration method but somewhat lower than values obtained by the Roe method. Likewise, Wollenberger (346) has investigated the possibility of estimating thiamine by polarographic methods. This author reports the formation of a characteristic wave at the dropping mercury electrode which he attributes to thiamine and which is said to be a function of the amount of thiamine present.

Winzler *et al.* (347) claim that the sensitivity of the colorimetric method used in determining choline is enhanced by using ultraviolet light instead of visible light, the choline reineckate in acetone manifesting intense adsorption at 327 mμ. The authors claim an accuracy of ±5 per cent with samples containing from 50 to 400 μg. of choline chloride.

MICROBIOLOGICAL METHODS

Cannon, Boutwell & Elvehjem (348), employing microbiological assay procedures, investigated the vitamin content of five different caseins, i.e., crude, alcohol extracted, acid washed, "Labco," and "Smaco." All five caseins were found to contain traces of vitamins. "Purified" casein was found to contain from one-tenth to one-half the vitamin content of the crude casein. Acid-washed caseins were found to contain somewhat less riboflavin than nonacid-washed casein. The authors concluded that sources of complete protein, free from growth-stimulating substances, are not available for experimental purposes. Johnson (349) has proposed a method for the determination of nicotinic acid, nicotinamide, and nicotinuric acid in solutions containing one or more of these compounds. The method is based on the finding that the organism, *Leuconostoc mesentoides,* which requires nicotinic

acid for growth, is unable to utilize nicotinamide or nicotinuric acid at the levels of assay investigated.

Melnick *et al.* (350) have published a critique of the methods employed in determining the vitamin B_6 complex and its components. These authors reaffirm and discuss some of the difficulties encountered in carrying out differential assays for the members of the B_6 group. Snell (351) has observed that an enzyme-digest of vitamin-free casein contains a substance which, with *dl*-alanine, promotes the growth of *L. casei* in the absence of pyridoxine. In contrast to pyridoxine, this substance is destroyed by acid and alkali hydrolysis, is not destroyed by light, and is not readily absorbed on charcoal. With the factor present in optimal amounts, the response of *L. casei* to alanine can be determined. Under these conditions $d(-)$alanine promotes growth while $l(+)$alanine exhibits no growth-stimulating properties. With *S. faecalis* R as the test organism, alanine will also replace pyridoxine in the medium, but $d(-)$alanine is reported as being most effective in this respect. In the presence of pyridoxine, $dl(-)$alanine appears to be the most active of the two isomers. Sarett & Cheldelin (352) have suggested a method for assaying the lactone moiety of pantothenic acid which is based on the growth response of *Acetobacter suboxydans,* while Hoag, Sarett & Cheldelin (353) have recommended that *L. arabinosus* be used as the test organism when assays are made for the whole pantothenic acid molecule. These investigators prefer *L. arabinosus* to *L. casei* in that its response is greater and more rapid in the effective range of concentrations.

Hofmann & Winnick (354) have described a method for estimating the oxybiotin content of biological materials. The procedure is based on the destruction of biotin by dilute potassium permanganate and the stability of oxybiotin to this reagent. Evidence is advanced to show that *Saccharomyces cerevisiae* and *Rhizobium trifolii* utilize oxybiotin and do not convert it to biotin. These authors state that "the sulfur atom is not essential for the biological activity of biotin."

Teply & Elvehjem (355) have described a titrimetric method for the determination of the "*L. casei* factor" and "folic acid." The method is based on a modification of the *S. lactis* R medium of Luckey (356) so as to promote more rapid growth and greater acid production, thus making conditions more favorable for use in the titrimetric assay of folic acid. These authors recommend that crystalline B_c be used as the standard. Bird *et al.* (357) have described a microbiological procedure which has been used successfully in assaying the

vitamin B_c conjugate. The method depends on the breaking up of the conjugate with a suitable enzyme and determining the free vitamin, using *L. casei* as the test organism. On the whole, *L. casei* assays of natural products, following enzyme digestion, yielded data which were in good agreement with those obtained by chick antianemia assays. The agreement in results was less satisfactory, however, when certain plant extracts were assayed by the two methods. Krueger & Peterson (358) have investigated some of the dietary factors which complicate vitamin B_c (folic acid, *L. casei* factor) assays when carried out with *L. casei* or *S. faecalis* as test organisms. These authors conclude that vitamin B_c (from liver and from yeast) has the same potency when measured by the two organisms, but that the differences in assay values were due to contaminants. Thymine was found to completely replace vitamin B_c in the nutrition of *S. faecalis* but not in the nutrition of *L. casei*. As little as one microgram of thymine caused errors of considerable magnitude. Siegel (359) has proposed a modification of the Horowitz & Beadle microbiological method for estimating choline, which is claimed to offer definite advantage. However, the principal innovation seems to have been the substitution of a fritted glass filter for the filter paper as suggested by the original investigators.

Leonian & Lilly (360) have made a comprehensive study of the comparative value of different test organisms in the microbiological assay for the B-vitamins. They found that the vitamin potency of yeast varied widely with the type of organism used in the assay but that the differences could be explained on the basis of the differences in the availability of the respective vitamins to the different test organisms. Wood (361) has published a detailed discussion of the inherent errors in the usual procedure of calculating vitamin concentration on the basis of the so-called "single curve" method. He shows that, mathematically as well as practically, much greater accuracy is obtained by using the "slope ratio" method which he describes with appropriate examples.

FOOD SOURCES

Natural Foods

Milk.—Lawrence, Herrington & Maynard (362) published an excellent summary of the literature on the various factors affecting the vitamin content of bovine and human milk. Mawson & Kon

(363) investigated the ascorbic acid content of milks as sold on the retail market in England. A total of 146 samples, consisting of 71 "raw" milks and 75 "heated" milks, were assayed. It was found that the raw milks contained, on the average, 1.34 mg. ascorbic acid per 100 ml., while the heated milks contained an average of 0.69 mg. ascorbic acid per 100 ml. The authors point out that the majority of the "heated" milks were pasteurized by the official British method which is known to destroy less ascorbic acid than was indicated in the above analysis. It is concluded that the unusually low ascorbic acid content of the heated milks was due to unsatisfactory handling methods. Munks and associates (364) have reported on the total ascorbic acid content of human milk. These authors found that human milk during the first ten days following parturition contained an average of 7.2 mg. total ascorbic acid per 100 ml., while milk samples taken at a later stage of lactation contained only 5.2 mg. ascorbic acid per 100 ml. In 35 per cent of the milk samples, dehydroascorbic acid was present to the extent of 14 per cent of the total ascorbic acid. In a continuation of their studies relative to the vitamin content of human milk, Lesher, Brody, Williams & Macy (365) reported carotene values of 25 μg. and vitamin A values of 60 μg. per 100 ml. for mature milk and a carotene value of 241 μg. and a vitamin A value of 198 μg. per 100 ml. for human milk taken the first day after parturition.

Holmes et al. (366) investigated the effect of sunshine on the ascorbic acid and the riboflavin content of milk. Milk in commercial one-half pint bottles was exposed to direct sunshine for from 30 to 60 minutes and the changes in ascorbic acid and in riboflavin were determined. The results show that practically all of the reduced ascorbic acid was destroyed during the first thirty minutes of exposure. While the riboflavin disappeared more slowly than the ascorbic acid, the rate of destruction of this vitamin was proportional to the intensity of irradiation. The authors conclude that commercial milk allowed to remain on the doorstep exposed to sunshine, loses appreciable amounts of ascorbic acid and riboflavin.

Holmes et al. (367) studied the ascorbic acid content of goat's milk. Thirty-nine samples of milk from four breeds of goats were investigated. Milks from stall-fed goats were found to be higher in riboflavin but lower in ascorbic acid than milks from goats receiving green feeds.

Roderuck et al. (368, 369) made a study of the thiamine and the riboflavin content of human milk as affected by diet and by

stage of lactation. Both free and combined vitamins were determined. Approximately 50 per cent of the thiamine content of human milk was found to exist in the free state while free riboflavin constitutes from 43 to 86 per cent of the total riboflavin present. These authors conclude that diet is the principal factor affecting the thiamine and the riboflavin content of human milk.

Coryell *et al.* (370) investigated the niacin, the pantothenic acid, and the biotin content of human colostrum and mature human milk. Milk from mothers, ten days after parturition, was found to contain 245 µg. of niacin per 100 ml., which was found to be higher than for mature milk from mothers on home diets. Mature milk from mothers receiving well-balanced diets averaged 196 µg. of niacin per 100 ml. Human colostrum was found to be low in niacin but the milk usually contained the normal content of this nutrient at about the tenth day. The pantothenic acid content of human milk was found to range from 48 µg. per 100 ml. on the first day to 245 µg. on the fourth day and occasionally to 304 µg. by the tenth day. The biotin content of human milk was found to be low during the first few days after parturition but increased until a normal value of 0.80 µg. per 100 ml. was attained.

Meats.—Rice & Beuk (371) studied the effect of cooking temperatures on the rate of thiamine destruction in pork and found that the rate of destruction was proportional to the cooking temperatures above 77°C. In further studies, Rice and co-workers (372) investigated the distribution of the B-complex vitamins in pork muscle tissues. Twenty-four tissues from several animals were assayed for thiamine, riboflavin, niacin, and pantothenic acid. It was found that different muscle tissues from the same animal varied as much as 300 per cent in vitamin content. Tissues high in thiamine were usually found to be high in niacin but low in riboflavin and in pantothenic acid. They postulate that muscle function and activity may be associated with variations in vitamin content.

Scrimshaw and co-workers (373), using three popular poultry breeds, studied the effect of genetic variation in the fowl on the thiamine content of the egg. They found that eggs from White Leghorn hens contained 270 µg., while eggs from Rhode Island Reds and from Barred Rocks contained 165 and 175 µg. of thiamine per 100 gm. of yolk, respectively. These authors attribute the high thiamine content of the White Leghorn eggs to the more efficient utilization of thiamine by this breed.

Peterson *et al.* (374) studied the riboflavin content of eggs, using a fluorometric method of assay. Eggs from the same bird were found to vary only slightly in riboflavin content from day to day while eggs from different birds were found to vary widely with respect to this vitamin.

Fruits.—Mustard & Lynch (375) studied the various factors influencing the ascorbic acid content of Florida-grown mangoes and concluded that variety of fruit was the major contributing factor so far as ascorbic acid content is concerned. Mangoes which had been sprayed with "Fermate" were reported to have more ascorbic acid than unsprayed fruit. Munsell (376) made an exhaustive study of the ascorbic acid content of a wide variety of Puerto Rican fruits and vegetables as determined by the dye-titration method. The study included oranges, grapefruit, limes, mangoes, papayas, avocados, pineapples, and other fruits. In general, guavas were found to be the richest source of this vitamin. The author concluded that there is no reason for any one to subsist on low ascorbic acid rations in Puerto Rico.

Hartzler (377) found the ascorbic acid in papayas and guavas to be just as available as crystalline ascorbic acid for human subjects. Stamberg (378) studied the ascorbic acid and the carotene content of two different varieties of rose hips and jams and juices made from each of these. Stability of the vitamins toward freezing and dehydration was also studied. Ripe rose hips were found to contain from 694 to 1124 mg. per cent of ascorbic acid and 19 to 28 mg. per cent of carotene. Jams made from the rose hips were found to be unusually rich in ascorbic acid which was well retained during storage. Juices prepared from rose hips contained more ascorbic acid than tomato juice or orange juice.

In their studies relating to the vitamin content of fruits and vegetables, Smith & Caldwell (379) found that reduced ascorbic acid in certain products was converted to the reversibly oxidized form so rapidly during blending that it was necessary to determine the total ascorbic acid content in evaluating the nutritive value of these products. Bauernfeind & Siemers (380) have suggested the use of ascorbic acid in the preservation of natural color in frozen peaches. Ascorbic acid is said to serve as an excellent antioxidant in the preservation of color in this fruit. The amounts of ascorbic acid required and methods of application are described.

Vegetables.—Kemmerer and co-workers (381, 382) investigated

the constituents of the crude carotene of some human foods. These investigators found that 76 per cent of the crude carotene from leafy vegetables was in the form of β-carotene, while raw carrots contained 62 per cent, raw sweet potatoes 86 per cent, and raw squash 51 per cent of the total carotene in the beta form. The crude carotene content of fresh carrots was found to range from 386 to 1120 p.p.m. Harper & Zscheile (383) investigated the carotenoid content of sixteen varieties and eighteen strains of carrots, using chromatographic and spectrographic means of analysis. Garden varieties of carrots were found to average 54 μg. of carotene per gram of material, of which 46 per cent was α-carotene. ζ-Carotene was found to occur rather generally in a large number of the varieties while lycopene was found to be present only in certain varieties.

Hamner *et al.* (384, 385) studied the influence of a number of environmental factors on the ascorbic acid content of tomatoes. Data are reported which show that the ascorbic acid content of the tomato is only slightly influenced by the degree of ripeness after the fruit is once mature, by storage, by fertilizer treatment during the growing season, or by humidity. Light intensity during the ripening stage was found to have the greatest influence on ascorbic acid content. Very little loss of ascorbic acid was detected during picking or during transportation of the fruit to the cannery. Holmes and co-workers (386) investigated summer squashes with respect to their vitamin content. Seven varieties of squash were assayed for ascorbic acid, carotene, and riboflavin. The results show that all varieties of squashes contained measurable quantities of the several vitamins.

Lampitt *et al.* (387) investigated the ascorbic acid content of the potato with respect to the distribution of the vitamin in the tuber as well as with respect to the influence of variety, soil types, and storage conditions on the ascorbic acid content. Their results show that the ascorbic acid content of potatoes was not affected appreciably by variety or soil type. Ascorbic acid was found to disappear rapidly during storage, unless sprouting occurred. During sprouting, an increased concentration of ascorbic acid was observed around the eye and in the sprout. No evidence of ascorbic acid localization was found in the unsprouted potato. Likewise, Murphy and co-workers (388) have investigated the genetic, physiological, and environmental factors affecting the ascorbic acid content of Maine-grown potatoes. A total of fifty-four commercial varieties were assayed for ascorbic acid, by means of a modification of the usual dye-titration method of assay.

The ascorbic acid content did not appear to be influenced by the size of the tuber but different varieties were found to contain from 20 to 45 mg. of ascorbic acid per 100 gm. of potato.

Gordon, Griswold & Porter (389) have investigated the effect of snow-ice on the retention of ascorbic acid in green vegetables displayed in a retail store. Vitamin assays were conducted on leaf lettuce, green beans, and spinach as purchased in the morning and after eight hours on an iron rack or in a Belshaw snow-iced display unit. It was reported that the snow-ice system of vegetable storage was effective in preserving the vitamin and in retaining the fresh appearance of the several products.

Van Duyne, Chase & Simpson (390) studied the effects of peeling, holding, and various methods of cooking on the ascorbic acid content of several varieties of potatoes. According to the data reported, ascorbic acid retention was reasonably satisfactory under the experimental conditions described.

Zozaya & Zuniga (391) investigated the niacin content of a wide variety of Mexican foods. The study involved seventy-seven vegetables and fourteen meats.

Cereals.—Bayfield & O'Donnell (392) determined the thiamine content of wheat after varying periods of storage, ranging from five months to fifty-one years. Their results show that there is a marked loss of thiamine from wheat even under normal storage conditions. With abnormal storage conditions, such as high moisture and high temperature, the rate of thiamine loss is greatly accelerated. Miller (393) determined the thiamine content of Hawaiian cereals after treatment with carbon disulfide or with methyl bromide to prevent insect infestation. No significant effect on the thiamine content of the wheat could be noted.

By means of a special technique, Somers *et al.* (394) studied the distribution of thiamine and riboflavin in the wheat kernel. Thiamine was found to be localized primarily in the aleurone layer, in the endosperm cells adjoining the aleurone layer, in the scutellum of the embryo, and in the endosperm adjoining the scutellum. By a somewhat different technique it was found that riboflavin was located in greatest amounts in the embryo and in the aleurone layer. Daniel & Norris (395) investigated the thiamine, riboflavin, and niacin content of dried leguminous seeds. From their data they conclude that the legumes are good sources of all three vitamins. Peanuts were reported as being an excellent source of niacin.

Processed Foods

Milk.—Berl & Peterson (396) studied the distribution of carotene and vitamin A in skim milk, butter, and buttermilk in butter-making. They were able to account for slightly more than 100 per cent of the vitamin present in the fresh milk, of which more than 90 per cent was found in the butter. No attempt was made to differentiate between the carotene and the noncarotene carotenoids.

Jenness & Palmer (397) investigated the vitamin A potency of more than one thousand samples of creamery butter produced in Minnesota. These authors found the vitamin potency to range from 9000 I.U. per pound in winter butter to 18,500 I.U. for summer butter. Carotene was found to account for 15 per cent of the potency of winter butter and 25 per cent of the potency of summer butter. Holmes *et al.* (398) studied the effect of high-temperature-short-time pasteurization on the ascorbic acid, thiamine, and riboflavin content of milk. Twenty lots of milk were investigated over an eighteen-month period. The results showed that this type of pasteurization had little or no effect on the vitamin content of milk. Holmes *et al.* (399) also investigated the vitamin content of chocolate milks. Chocolate milks prepared by the American process were found to be superior in vitamin content to other chocolate milks prepared according to the Dutch process. Shetlar, Shetlar & Lyman (400) measured the riboflavin content of chocolate and whole milk. It was found that whole milk loses its riboflavin content much more rapidly than chocolate milk when exposed to sunlight for the same period of time. Theophilus & Stamberg (401) investigated the influence of breed, feed, and processing on the riboflavin content of milk. Milks from Jersey cows were found to contain about one-third more riboflavin than comparable milks from Holstein cows. The riboflavin content of the milk was found to be influenced strikingly by the diet of the cow but stage of lactation, pregnancy, and estrus had no apparent effect on riboflavin content. Colostrum was found to contain three times as much riboflavin as normal milk but the high concentration of the vitamin soon disappeared following parturition. Hodson (402) has reported data on the niacin, pantothenic acid, choline, and biotin content of fresh, irradiated, evaporated, and dried milk. The data tend to show that there were no significant losses of these vitamins from the milks resulting from any of the above mentioned treatments.

Meats.—Jackson and co-workers (403) investigated thiamine,

riboflavin, and niacin retention in various cuts of fresh, cured, and cooked pork. With the exception of the tenderloin, there were greater variations in the vitamin content among different carcasses than there were among different cuts from the same carcass. Cooking had an adverse effect on vitamin retention. The greatest loss of thiamine occurred during roasting and frying, while riboflavin and niacin were not seriously affected by these methods of cooking. Greater loss of thiamine occurred during roasting than during frying. "Dry-cure" favored retention of thiamine and niacin, while "wet-cure" favored retention of riboflavin.

Sarett & Cheldelin (404) studied thiamine, riboflavin, and niacin retention in connection with the preparation of overseas hams and bacon. It was found that from 70 to 80 per cent of the vitamin content of hams was retained after soaking and boiling. However, the loss of vitamins from the bacon, as the result of soaking, was considerably higher than from the hams. More extensive vitamin losses were sustained during cooking than during soaking. Here again vitamin retention in hams was greater than in bacon.

Cruickshank et al. (405) studied the vitamin content of spray-dried eggs during dehydration and subsequent storage. It was found that there were no losses of vitamins A and D during drying and that only 30 per cent of the thiamine could not be accounted for. During storage, there were no losses of vitamins A, D, and thiamine when the dried eggs were stored at 15°C. in an atmosphere of nitrogen. High storage temperature was found to be more detrimental to thiamine retention than increased moisture content. Riboflavin and niacin retentions were satisfactory under all conditions of storage.

Pugsley et al. (406) made a study of the variability of the vitamin A and D potencies of the liver oils of codfish landed at ports of Nova Scotia, New Brunswick, and the Gaspe Peninsula of Quebec. A study was also made of the factors influencing the above-mentioned variations. These investigators found that with an increase in vitamin A potency there was always a parallel increase in vitamin D potency. Livers of cod caught in the Spring contained less oil, but the oil was of higher vitamin potency than liver oils taken from cod caught in the Fall. The product of the volume of the liver oil times its vitamin potency was found to approximate a constant. Liver oil from large cod was found to have higher vitamin content than oil from small cod. Hoar & Barberie (407), using the microbiological method of assay, investigated the riboflavin content of one hundred and twenty-five

samples of fish. Fish muscle was found to vary in riboflavin content, but, on the average, contained less of this vitamin than meat. Glandular organs of fish were found to contain less riboflavin than corresponding glandular organs from mammals. The riboflavin content of fish tissue was not affected by the size of the fish. No appreciable losses of riboflavin resulted from freezing or smoking of fish but canning, salting, and drying caused appreciable loss of this vitamin.

Fruits.—Moore, Wiederhold & Atkins (408) studied the retention of ascorbic acid in canned Florida grapefruit juices during storage. The results show that the average ascorbic acid retention of canned unsweetened grapefruit juice from twelve central Florida canneries was 95 per cent at the end of two months, 90 per cent at the end of four months and 83 per cent at the end of six months' storage. Lincoln & McCay (409) studied the retention of ascorbic acid in marmalade during preparation and storage. It was found that citrus marmalade retained about 68 per cent of the ascorbic acid originally present in the ingredients. When stored at 38°F. marmalade showed good retention of ascorbic acid but at higher temperatures the vitamin disappeared rather rapidly.

Since black currant syrup was an important source of ascorbic acid in England during the war, Kendrick & Downer (410) initiated a study of methods of preserving the vitamin content of the fruit so that syrup-making need not be restricted to the relatively short black currant picking season. The investigators found that it was practical to preserve the fruit with sulfur dioxide without appreciable losses of ascorbic acid. In fact the loss of vitamin was no greater than would have been the case if the syrup had been made in season and stored for a similar period. Perlman & Morgan (411) studied the stability of the B-vitamins in grapefruit juices and wines and found that these products retained the added vitamins, thiamine, riboflavin, pyridoxine, and pantothenic acid, quite satisfactorily during storage, except that riboflavin, natural and added, was largely destroyed when these products were bottled in clear glass.

McConnell, Esselen & Guggenberg (412) investigated the effect of storage conditions and type of container on the stability of carotene in canned vegetables (asparagus, green beans, corn, and peas) and found the provitamin to be relatively stable during canning and storage in commercial types of glass and metal containers. Bailey & Dutton (413) investigated the apparent increase in the carotene content of carrots during dehydration but failed to detect the presence of

any new pigment. They conclude that the observed increase in caro-
tene is not a function of systematic error in the carotene determina-
tion, but that it is a direct result of the loss of soluble solids during
dehydration. Lee & Whitcombe (414) found that the nature of the
water used in blanching peas and snap beans had no significant effect
on vitamin retention. Wagner *et al.* (415) studied the effect of com-
mercial canning on the ascorbic acid content of grapefruit juice as
prepared in twelve different canneries located in the Rio Grande
Valley. These authors reported ascorbic acid retention ranging from
92.0 to 99.6 per cent after canning. During subsequent storage for
forty days at 72°F., 91.3 per cent of the ascorbic acid was found to
remain in the product.

Vegetables.—Retzer *et al.* (416) studied the effect of steam and
hot water blanching on the ascorbic acid content of snap beans and
cauliflower. Snap beans were found to lose 15 per cent of the vitamin
through water blanching and 11 per cent through steam blanching.
When ready to freeze, cauliflower had lost 19 per cent of its ascorbic
acid as a result of water blanching and 18 per cent as a consequence
of steam blanching. After nine months of frozen storage, both prod-
ucts had lost 60 to 70 per cent of the ascorbic acid. Wadsworth &
Wilcox (417) reported that lima beans lose about 28 per cent of their
ascorbic acid content as the result of a two-and-a-half-minute blanch
and that only 49 per cent of the original ascorbic acid remained after
canning. These authors claim greater retention of ascorbic acid by
cooked frozen lima beans than by cooked canned beans. Lee & Whit-
combe (418) investigated the ascorbic acid content of frozen and
cooked soybeans and found that the green beans and the bean sprouts
had sustained considerable loss of vitamin as the result of blanching,
freezing, and cooking.

According to the report of Cohee & Goodale (419), plain and
fortified tomato juices lose approximately the same percentage of
ascorbic acid during storage. The addition of sucrose had no bene-
ficial effect on ascorbic acid retention by this product. Robinson,
Stotz & Kertesz (420) studied the effect of manufacturing methods
on the ascorbic acid content of tomato juice. Under efficient manu-
facturing conditions, these investigators found tomato juice to have
retained 80 per cent of the original ascorbic acid. These authors state
that in order that tomato juice contain 20 mg. of ascorbic acid per
100 ml. of juice as required by the Council on Foods and Nutrition
of the American Medical Association, the tomatoes from which the

juice is prepared must contain not less than 25 mg. of the vitamin per 100 gm. of trimmed fruit. Moyer & Stotz (421) have reported negligible loss of ascorbic acid from tomato juice when electronic blanching is used in contrast to losses of 30 to 40 per cent of the vitamin when steam or water blanching are employed.

Noble & Waddell (422) investigated the ascorbic acid content of different varieties of cabbage before and after cooking by various methods. Fresh cabbage was found to contain from 36 to 49 mg. of the vitamin per 100 gm., depending primarily on the variety of cabbage. The percentage of vitamin retained by cooked cabbage was found to be variable and not dependent on cooking time. Cabbage cooked in a covered kettle was found to retain from 46 to 67 per cent of the ascorbic acid, while, with open kettle cooking, only 30 to 34 per cent of the vitamin was retained. Hollinger & Colvin (423) investigated the ascorbic acid content of two varieties of okra as affected by maturity, storage, and cooking. Young okra pods were found to be a good source of ascorbic acid but, as the pod increased in maturity, the vitamin content was found to decrease. The retention of ascorbic acid in cooked okra was greatest when cooking was done in a covered pan.

Eheart & Sholes (424) investigated the effect of the method of blanching, storage, and cooking on the ascorbic acid content of dehydrated green beans. According to the report of these authors, blanching has no significant effect on the ascorbic acid content of green beans but blanched beans contain more ascorbic acid after dehydration than unblanched beans. The method of storage of the dehydrated beans was also found to affect ascorbic acid retention. Harris (425) has reported that salted vegetables lose carotene and ascorbic acid more rapidly than unsalted vegetables. Morgan et al. (426) investigated the retention of ascorbic acid, thiamine, riboflavin, pantothenic acid, and niacin in carrots, spinach, broccoli, green peas, and snap beans as the result of blanching, dehydration, and cooking. Riboflavin, pantothenic acid, and niacin were found to be relatively stable during blanching, dehydration, and cooking. Losses of ascorbic acid and thiamine were greater from unblanched vegetables than from blanched vegetables.

In a comparison of the thiamine content of home-canned and commercially canned tomato juices, Poe & McGuire (427) found commercially canned juices to contain the greatest amount of the vitamin. Miller (428) investigated the thiamine content of Japanese soybean

products and concluded that these products are poor sources of this vitamin, owing to extensive losses during preparation. Pearson & Luecke (429) studied the B-vitamin content of four varieties of sweet potatoes before and after cooking and also before and after sprouting. These authors found that sweet potatoes, which had been baked, retained 76, 89, 85, and 77 per cent, respectively, of thiamine, riboflavin, niacin, and pantothenic acid. Boiled sweet potatoes, on the other hand, were found to retain 92, 103, 101, and 100 per cent, respectively, of thiamine, riboflavin, niacin, and pantothenic acid. Where there was some thiamine loss on sprouting, the riboflavin, niacin, and pantothenic acid content of sprouted sweet potatoes and of sweet potato sprouts was essentially the same as that of the unsprouted potatoes.

Hinman, Brush & Holliday (430) investigated the retention of ascorbic acid, thiamine, and riboflavin in small-scale preparation of typical canned foods. Two methods of preparation were studied: (a) where the liquid in the can was concentrated and retained, and (b) where the liquid was drained off and discarded after the product had been heated. While ascorbic acid retention was found to be variable, the loss in vitamin was always greatest where the liquid was discarded. There were insignificant losses of thiamine and riboflavin due to destruction but from 30 to 40 per cent of these vitamins were lost when the liquid was discarded. Guerrant, Vavich & Dutcher (431) investigated the stability of the vitamins of canned foods as affected by time and temperature of storage. The vitamin content of canned tomato juice, green lima beans, and whole-kernel yellow corn was determined at regular intervals during storage for one year at temperatures ranging from 30°F. to 110°F. All vitamins were found to be affected adversely by prolonged storage at the higher temperatures, the amount of vitamin retention depending on the vitamin and the type of product. From the standpoint of vitamin retention, the most satisfactory storage temperature for canned foods was indicated as being 42°F. or lower. Ives et al. (432) assayed thirty-six commercially canned foods for riboflavin, niacin, and pantothenic acid and found the vitamin content of the 1943 pack to compare favorably with those foods packed and assayed during the previous season. Fenton et al. (433) studied vitamin retention in dehydrated potatoes after rehydration and after various methods of cooking. According to these investigators there appeared to be no significant losses of thiamine, riboflavin, and niacin as the result of rehydration and

cooking, but the ascorbic acid of potatoes was found to be very susceptible to destruction during the beating in the preparation of mashed potatoes.

Cereals.—Robinson and co-workers (434) made a study of the thiamine content of a large number of breakfast foods. Oatmeal, whole wheat cereal, and those made of a mixture of several cereals were found to be highest in thiamine content. A single serving of many of the cereals contributes from 10 to 15 per cent of the recommended daily allowance of this vitamin. Corn, wheat, and rice endosperm types of cereals were found to be low in thiamine. Loss of thiamine from breakfast cereals due to storage and cooking was found to be small. Eklund & Goddard (435) found no loss of thiamine in fortified cereal as the result of cooking. Meckel & Anderson (436) measured the retention of thiamine in four types of army bread made from enriched flour. From 76 to 86 per cent of the thiamine was found to be retained after baking. These losses are in agreement with those reported from commercially baked breads made from enriched flour.

Westerman & Bayfield (437), using the biological method of assay, investigated enriched, Morris type, and whole wheat flour as sources of the B-complex vitamins. These authors interpret their data as indicating that whole wheat flour is more efficient in promoting growth in rats than is the Morris type of flour or the patent flour enriched at the old level. Whole wheat flour and the new enriched flour promoted the same amount of growth when fed at the rate of 40 per cent of the diet but, when fed at the rate of 50 per cent of the diet, the new enriched flour proved to be a better source of the B-vitamins than the whole wheat flour.

Younger & Harvey (438) investigated the stability of added thiamine, riboflavin, and niacin in beer during storage in clear and in amber bottles, when exposed to light and when stored in the dark. It was found that beer stored in clear bottles and exposed to light lost 29 per cent of its thiamine in six months and 50 per cent of its riboflavin in one week. There was no appreciable loss of niacin during six months of storage. In an investigation of vitamin losses from deep-fat cooking of doughnuts, Everson & Smith (439) found that about 23 per cent of the thiamine and 20 per cent of the niacin could not be accounted for, but there was no loss of riboflavin.

Spinella (440) described a type of sweetmeat containing brewers' yeast which was highly nutritious and was said to be well received by

factory workers. Its chemical composition and vitamin content are listed and discussed.

Bradford & Hughes (441) have investigated the riboflavin and the pantothenic acid content of tea. These authors report that an ordinary infusion of 5 to 6 oz. of tea usually contains about 25 μg. of riboflavin and 75 μg. of pantothenic acid.

Teply, Krehl & Elvehjem (442) have reported that roasted coffee contains about 10 mg. of niacin per 100 gm. and that restaurant coffee contains about 1 mg. per cup (175 ml.).

Hoffman *et al.* (443) observed high carotene retention in alfalfa meal when stored in an atmosphere having a low oxygen content. Mills & Hart (444) investigated the stability of carotene in dehydrated feeds and foods and found sliced dehydrated sweet potatoes lost 5 per cent of the carotene in six months' storage while finely ground dehydrated sweet potatoes lost as much as 65 per cent of the carotene while stored under similar conditions for the same period. Further evidence regarding the effect of exposed surfaces on susceptibility to increased oxidation of carotene was demonstrated when it was shown that carotene losses were greater in sliced carrots than in diced carrots. In a more recent report, Mills & Hart (445) concluded that the amount of surface exposed to oxygen is an important factor in determining the rate of carotene destruction in foods and feeds.

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DEPARTMENT OF AGRICULTURAL
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THE CHEMISTRY OF THE HORMONES

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Although some very important publications concerning the chemistry of the hormones appeared during the past year, the volume of the pertinent literature was small. The chemistry of the steroids, the physiologic actions of hormones and their metabolism will be discussed by others; hence this chapter deals merely with the chemistry of the nonsteroid hormones and only cursory reference will be made to allied fields.

PITUITARY

Young (1) has reviewed the functions of the anterior pituitary gland and Houssay (2) described the relation of this endocrine organ to experimental diabetes.

Lactogenic hormone.—Studies concerning the reaction between ketene and lactogenic hormone in buffer solutions of pH 4.0 and pH 7.0 at 0° C. showed that an acetylated lactogenic hormone exhibits a diminished crop-sack stimulating action in pigeons. From this it was concluded that the amino groups are essential for the biologic activity of the hormone (3). The relative viscosity of lactogenic hormone solutions has been determined in the presence of urea and of a detergent (Nacconol). An increase in viscosity was found to coincide with a lowering of biologic activity (4). The factors influencing the reliability of the biologic assay of lactogenic preparations have been analyzed (5, 6) in pigeons by the crop-sack weight method.

Thyrotropic hormone.—A procedure for obtaining highly purified thyrotropic preparations has been described in detail (7). One μg. of the material thus obtained produced definite histologic changes in the thyroids of three-day-old male white Leghorn chicks; it is free of prolactin, gonadotropic and growth activity. The thyrotropic hormone content of the anterior pituitary of female albino mice was found to be higher than that of males at twenty-six to twenty-seven and forty

to forty-one days, but lower at eighty-two to eighty-three days of age (8). A new cytologic method for the determination of the thyrotropic hormone has been described (9).

Adrenocorticotropic hormone.—The adrenocorticotropic hormone was found to control the rate of release of serum globulins and antibody from lymphoid tissue (10, 11, 12). Experiments on rats and rabbits revealed that single injections of adrenocorticotropic hormone, or adrenal cortical extracts, result in an increase in total serum globulin concentration. It was concluded that the lymphocytes in lymphoid tissue are a storehouse for a portion of the globulin fraction of the serum, and that the rate of release of this protein is under the normal physiologic control of the adrenocorticotropic hormone, which exerts its influence by way of the adrenal cortex. Apparently the adrenotropic hormone, through its effect upon the adrenal cortex, regulates serologic defense reactions which depend upon the antibody release from lymphatic tissue. It was also found that injection of adrenocorticotropic hormone exercises a beneficial effect upon the resistance of normal male rats to low atmospheric pressure (13).

In vitro, the brain tissue of mice, which were injected with adrenocorticotropic hormone daily for fifteen days, synthesized, on the average, 50 per cent more than the normal amount of acetylcholine (14).

Investigations concerning the effect of ketene, nitrous acid, formaldehyde, and iodine on the adrenocorticotropic hormone indicate that both the free amino and the tyrosine groups are essential for its action (15).

Pharmacologic studies imply that the anterior lobe contains and produces a hormone which resembles desoxycorticosterone acetate in that it causes nephrosclerosis and hypertension in experimental animals. It has not yet been possible to determine with certainty whether or not the action of this principle is due to the corticotropic hormone content of the anterior lobe extracts but preliminary experiments make this seem very probable. Apparently, a corticotropic hormone acts upon the adrenals and stimulates the latter to secrete mineral-active corticoids, which in turn cause hypertensive renal disease (16, 17, 18). The nephrosclerosis produced by anterior pituitary extracts (19), just as that due to desoxycorticosterone (20), can be inhibited by the administration of "acidifying" salts such as ammonium chloride or calcium chloride. High carbohydrate diets likewise counteract the nephrosclerotic action both of pituitary extracts and of desoxycorticosterone acetate (21).

Pharmacologic investigations have led to the assumption that there are several adrenocorticotropic hormones which enable the organism to stimulate, selectively, the production by the adrenal cortex of corticoids with qualitatively different effects as well as of testoid hormones. In order to avoid lengthy and complicated descriptions, the various hormones of the adrenal cortex have been designated as gluco-corticoids (causing hyperglycemia and glycogen deposition), lipo-corticoids (causing fat deposition especially in the liver), mineralo-corticoids (causing sodium retention and the restoration of the subnormal blood sodium and blood chloride levels in adrenalectomized animals), and testoids of the adrenal cortex (virilizing or "androgenic" compounds produced by the adrenal). The corresponding tropic hormones of the pituitary may then conveniently be termed gluco-corticotropins, lipocorticotropins,   mineralo-corticotropins,   and   testo-corticotropins (22).

Somatotropic (growth) hormone.—Li, Evans & Simpson (23) have described the isolation of the anterior hypophyseal growth hormone. The procedure consists of the following steps : extraction of the glands with calcium hydroxide, precipitation with ammonium sulphate, sodium chloride fractionation, additional fractionation by pH variation and ammonium sulphate, and finally by isoelectric precipitation. From osmotic pressure determinations, the molecular weight was found to be 44,250. Electrophoresis experiments gave an isoelectric point at pH 6.85. The following amino acids were determined : tyrosine 4.30 per cent, tryptophane 0.92 per cent, and glutamic acid 13.40 per cent. Cysteine was apparently not present. The hormonal activity was destroyed by pepsin and trypsin and was found to be more stable in alkaline than in acid medium.

Renotropic effect.—Under certain experimental conditions, crude anterior lobe extracts cause a pronounced enlargement of the kidney. This is due to hypertrophy and hyperplasia of the renal tubule cells as well as to an increase in the diameter of the glomeruli. This "renotropic effect" is greatly enhanced by the simultaneous administration of thyroxine (24) and diminished, but not abolished, by thyroidectomy (25). The increase in kidney size is much greater than could be expected on the basis of the growth hormone content of such preparations, and purified growth hormone, lactogenic, gonadotropic, and adrenotropic hormone preparations of anterior lobe tissue fail to exhibit any significant renotropic potency. Thyrotropic preparations are active, yet the effect does not appear to be due solely to thyroid

stimulation since it persists, to some extent, after ablation of the thyroid (26).

Insulotropic hormone.—Conn & Louis (27) claim to have obtained evidence for the presence in the anterior pituitary of an insulotropic principle which directly stimulates the islets of Langerhans. It is difficult to reconcile this view with the fact that hypophysectomy fails to cause any significant involution of the Langerhans islets.

Gonadotropic hormones.—The gonadotropic hormone content of cockerel anterior pituitaries has been determined, using the ninety-six-hour chick test. Potency for whole glands ranged from 0.5 chick unit on the fifth day to 8.3 units on the ninetieth day (28). The rate of inactivation of the physiologic activity of chorionic, pregnant mare serum, and sheep pituitary gonadotropins has been studied in a 40 per cent urea solution at 37.5° C. The inactivation of chorionic gonadotropin proceeded rapidly and was independent of the hormone concentration. Pregnant mare serum gonadotropin manifested a remarkable degree of stability. The inactivation of sheep pituitary gonadotropin proceeded less rapidly than that of chorionic gonadotropin (29). Study of the temperature coefficient for the denaturation of chorionic gonadotropin by urea revealed that this hormone is rapidly inactivated at 100° C., due to denaturation rather than oxidation (30). A chorionic gonadotropin preparation, partially inactivated by heating at 63 to 64°, could not be reactivated by hydroquinone (31). It has been reported that the diastase present in crude chorionic gonadotropic preparations is an impurity derived from the starting material and not a constituent of the hormone (32).

The activity of the gonadotropin in the dialyzed urine of castrated women could be augmented by the addition of a dialyzable substance present in the same urine (33). The effect of pituitary gonadotropin could be enhanced by administrating it combined with tannic acid (34) or certain metallic hydroxides (35).

Several modified procedures for the biologic assay of gonadotropic activity in urine of pregnancy have been published (36, 37, 38). The chorionic gonadotropin level in the serum appears to reach its peak between fifty and sixty-five days of normal pregnancy (39). There is no reliable chemical method available, as yet, for the determination of pregnancy (40). The possible presence of an ovulating factor in plant juice has been described (41).

Hormones of the pars intermedia and neuralis.—The oxytocic and pressor principles were separated from aqueous extracts of desiccated

posterior lobes by adsorption on permutit. The pressor principle is adsorbed, whereas the oxytocic factor is not. Elution of the pressor substance can be accomplished with sodium chloride solution (42).

Croxatto and his associates have studied the effect of various enzymes on vasopressin and oxytocin. Aminopolypeptidase from yeast and hypertensinase from kidney destroy the vasopressor and oxytocic principles in the presence of cysteine or glutathione (43, 44). Chymotrypsin destroys both hormones; trypsin inactivates the pressor but not the oxytocic principle (45). Carboxypeptidase has no effect on vasopressin or oxytocin (46), although tyrosinase destroys the oxytocic effect (47). The oxytocic principle reacts in two ways with formaldehyde. With a small quantity of formaldehyde an almost instantaneous reaction occurs both at 5° and at 38°C. and destroys about 20 per cent of the activity. There also is a slow reaction, the velocity of which is a function of both temperature and formaldehyde concentration (48).

Tyrosine, arginine, cystine (49) and tryptophane (50) have been shown by Stehle to be constituents of the melanophore homone. A method, accurate to within 10 per cent, has been described (51) for the assay of the melanophore-expanding principle, *Xenopus laevis* being used as a test animal. The criterion of potency is the maximal melanophore index attained after injection of extracts into the dorsal lymph sacs of fully pale intact or hypophysectomized animals. Antidiuretic material in tissues, blood, and urine has been estimated, employing dogs with experimental diabetes insipidus as test animals. Ratios of urine creatinine to plasma creatinine have been compared after intravenous administration of pituitrin and the unknown material (52). Using this test, the supraoptic region of the dog's hypothalamus was found to contain an amount of water-soluble antidiuretic substance equivalent to 1.5 to 5.0 units of pituitrin (53).

THYROID

In last year's volume, Salter (54) has given a comprehensive review of our present knowledge concerning the thyroid.

The consumption of oxygen, by slices of mature male guinea pig thyroids, was studied by the Warburg technique. The average oxygen consumption as cc. per gm. cell wet wt. per hr. at 5°, 38°, and 40° was 0.156, 1.037, and 1.889, respectively (55). An increase in the serum α-globulin fraction was found in hypothyroid rats (56). The response of the thyroid to thyrotropic hormone is less in immature

female guinea pigs injected with thyroxine daily for eleven days, than in untreated controls (57). The thyrotropic hormone content is diminished both in the serum and in the pituitaries of rats fed diets containing 1 per cent thiourea or sulfadiazine but is increased in thyroidectomized rats (58). A colorimetric method for the determination of iodine in rat thyroids has been developed (59).

Addition of hydrogen peroxide or of iodine accelerates the conversion of di-iodotyrosine to thyroxine and increases the yield of thyroxine. The actual agent, effective in bringing about the oxidation of di-iodotyrosine to thyroxine, appears to be free iodine (60). In this connection, reference may be made to studies concerning the catalytic effect of acetate and phosphate buffers on the iodination of tyrosine (61) and the iodination of tyrosine groups in serum albumin and pepsin (62). l-Thyroxine has been reported to be twice as active as dl-thyroxine when both were tested by their abilities to reduce the weights of the hyperplastic thyroids of thiouracil-treated chicks and rats, or to stimulate metamorphosis in frog tadpoles. Accordingly, all the activity of the dl-form is accounted for by its l-thyroxine content (63). Harington (64, 65) has reviewed the mechanism of the biosynthesis and the immunochemistry of thyroxine.

The claim that paraxanthine (1,7-dimethylxanthine) antagonizes the effect of thyroid hormone (66) could not be substantiated (67).

Houssay and his associates have presented new data on the relationship of thyroid activity to diabetes mellitus (68). Prolonged administration of thyroid was found to produce diabetes in partially depancreatized dogs. Diabetes so produced may be observed either only during the thyroid feeding (thyroid diabetes), or it may persist permanently even after stopping the treatment (metathyroid diabetes). In both cases, the beta cells of the islets are damaged, reversibly in the first, permanently in the second. However, thyroidectomy does not modify diabetes produced in dogs by total pancreatectomy.

The use of tracer elements, in thyroid research, has been reviewed (68a, 68b).

INSULIN

The optical-crystallographic properties of crystalline zinc insulin have been reviewed (69). Chymotrypsin inactivates insulin in vitro but does not prevent its hypoglycemic effect when the two substances are injected simultaneously into rabbits or dogs. Intravenous injection of chymotrypsin alone has no significant effect on the blood sugar of

these animals (70). By microbiological methods 13.4 per cent leucine (71, 72) and 17.5 per cent glutamic acid (73, 74) were found to be present in insulin. Attention has been drawn to the possible errors in the insulin assay technique which is based upon the production of convulsions in mice (75), and the cross-over method of insulin assay (76) has been further developed. Haist (77) has reviewed the factors which affect the insulin content of the pancreas.

Two modifications of protamine zinc insulin have recently been suggested. The first, containing about 25 per cent of the hormone in quickly absorbable form, and 75 per cent in precipitated, slowly absorbable form, is designated "modified protamine-zinc insulin" (78). The second form is a mixture of crystalline insulin and protamine zinc insulin (79). Several comparative clinical studies deal with more recently developed insulin preparations (78 to 82).

The inhibitory effect of anterior pituitary extract on hexokinase activity in the transformation of glucose to glycogen could be released by insulin (83).

ADRENAL

The *in vitro* oxidation by phenoloxidase of epinephrine and chemically related amines has been the subject of systematic investigations (84).

The relatively high activity of hog adrenal extracts (assayed by the work test of Ingle) is due to a high concentration of 11-oxygenated steroids. About six times as much 17-hydroxycorticosterone could be crystallized from this source as from an equal amount of beef adrenal extract. After removal of the 11-oxycorticoids by crystallization, there was prepared, from hog and beef adrenal extracts, a water-soluble fraction. This exhibited a relatively low activity by the work test but high activity by the growth-survival test. The activity of the so-called amorphous fraction does not seem to be due to any of the known biologically active corticoids (85). Intramuscular administration of pork adrenal extract tends to correct the blood-sugar abnormalities of Addison's disease more effectively than beef adrenal extract (86). The greater proportional effect of pork adrenal-cortex extract is apparently due to its higher content of 11-oxygenated steroids, such as 17-hydroxycorticosterone. Comparative bioassays of several adrenal cortical extracts were performed (87) by four methods: (a) growth and survival in immature adrenalectomized rats, (b) renal function in adrenalectomized dogs, as judged by blood-urea nitrogen

levels, (c) sodium retention in normal dogs, and (d) glycogen deposition in fasted adrenalectomized rats. Employing a modification of the last-named test, corticosterone and its 11-dehydro derivative proved to have about the same glycogenic potency, while 11-dehydro-17-hydroxycorticosterone is somewhat more potent, and 17-hydroxycorticosterone about 1.5 times more potent than corticosterone; desoxycorticosterone acetate is inactive at 1.2 mg. (88). In the work test, the activities of 17-hydroxycorticosterone, 17-hydroxy-11-dehydrocorticosterone, corticosterone, and 11-dehydrocorticosterone were found to be, respectively, 6.3, 5.0, 2.3, and 1.6 units per mg. Desoxycorticosterone acetate proved ineffective in doses of 5 mg. (89). The work performance of adrenalectomized-nephrectomized rats is not markedly improved by daily pretreatment with desoxycorticosterone acetate for seven days before adrenalectomy (90).

A procedure has been developed for the extraction, purification, and colorimetric assay of urinary substances chemically related to 11-oxycorticoids (91) and 11-oxycorticoids have been demonstrated in the urine of patients with adrenal cortical tumors and adrenal cortical hyperplasia (92, 92a).

Hormones of the Gastrointestinal Tract

This field has recently been reviewed by Greengard (93) and Houssay (94). The presence of a gastric secretory excitant (gastrin) in the pyloric mucosa of pigs has been confirmed (95). Parenteral administration of a mucosal extract of the upper intestine of swine appears to prevent the occurrence of gastro-jejunal ulcers in a high percentage of Mann-Williamson dogs. The protection afforded against ulcer is not limited to the period during which the extracts are administered but extends to periods as long as three years after cessation of treatment (96). The effect of prolonged administration of enterogastrone on the gastric secretion of normal and Mann-Williamson dogs has been studied (97).

Kidney

The renin-hypertensin system.—Unfortunately, there still is no uniform generally acceptable nomenclature for the various renal and blood factors involved in experimental hypertension.

According to earlier work, as reviewed by Fraenkel-Conrat (98),

renin, a renal substance acts proteolytically on hypertensinogen (renin activator), a blood globulin, to produce hypertensin (angiotonin) the actual pressor principle; the last-named is gradually inactivated by another blood and tissue component, hypertensinase (angiotonin inhibitor).

The enzymatic component of swine kidney, responsible for pepsinase activity, is not identical with the enzyme renin. This was demonstrated (99) by fractional denaturation of pepsinase, which leaves the renin activity of the sample practically unaltered. Pepsitensin and angiotonin are both inactivated when incubated with various amounts of crystalline pepsin but show a distinct difference in the magnitude of the proteolytic coefficients as well as the hydrogen ion optima for these reactions. Hence, it appears that pepsitensin and angiotonin are not identical and probably differ in the number, rather than in the nature, of the amino acid residues of which they are composed (100). Plentl & Page (101) have purified hypertensin (angiotonin) by precipitation as a silver salt in acid and neutral solutions, followed by removal of the silver with hydrogen sulfide. Hypertensin can also be precipitated with mercuric chloride in neutral solution. Histidine was the only basic amino acid that could be found in the hydrolysate of hypertensin; no tyrosine, phenylalanine, or dihydroxyphenylalanine could be detected. Further evidence was obtained for the presence of at least one free amino group associated with pressor activity. Highly purified preparations of hypertensin have been obtained (102) by means of chromatography and electrodialysis. The smallest dose having an appreciable effect on the blood pressure of a cat was approximately 0.5 µg. The preparation of human renin, hypertensinogen, and hypertensin can be accomplished as follows: fresh human kidneys are extracted with water at 5°, the filtrate is adjusted to pH 6.0 and 10 per cent trichloroacetic acid is added to pH 2.9. The hypertensinase is precipitated in the filtrate by adding a 5.13 M sodium chloride solution until the concentration is 0.92 M sodium chloride. The renin remains in solution and is recovered by precipitation with ammonium sulfate at pH 5.0 (103). The inhibition of hypertensinase activity can serve as a basis for the quantitative assay of renin substrate (104). Pepsitensin and hypertensin are readily inactivated by carboxypeptidase (105). The destruction of hypertensin by renal extracts (hypertensinases) is markedly accelerated by oxidized cytochrome and sharply retarded by reducing agents such as ascorbic acid and cysteine (106).

ANTIHORMONES

The present status of antihormones has recently been reviewed by Thompson (107) and Leathem (108).

Antigonadotropic substances could not be detected in the sera of patients injected with a combination of sheep anterior pituitary extract and chorionic gonadotropin. The maximum period of hormone administration was five months (109). However, it has been found that administration of a combination of sheep pituitary extract and chorionic gonadotropin produces antigonadotropins in rabbits (110) and that an antigonadotropic substance is detectable in rabbits following administration of a sheep pituitary extract (111).

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THE BIOCHEMISTRY OF TEETH

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During the period covered by the first of these reviews in 1934 (1), little basic work had been done on the biochemistry of the teeth. The period covered by the second review in 1942 (2) was one of great advance in knowledge of the physical and chemical properties of teeth. The work reviewed in this paper is therefore based upon a much firmer biochemical foundation than were the earlier studies. The process of integrating a very large quantity of previously apparently unrelated data is now well under way, and holds much promise for the future.

The Mechanism of Tooth Formation

Early studies of enamel development were largely histological, but attempts have recently been made to correlate chemical and histological findings to produce an integrated picture of what is actually occurring.

Two stages in enamel formation have been clearly differentiated. In the first stage, the protein matrix is laid down. This soft, easily cut, and acid-insoluble material grows outward from the dentino-enamel junction and downward from the cusp (3). Even in this early stage the matrix contains some calcium salts, but they are apparently not deposited until the keratin-like protein has been formed by the ameloblasts (4). The protein after its deposition undergoes a gradual hyalinization (4, 5), a process which may continue even after the tooth has erupted (6). These changes have been compared to those characteristic of skin, another epithelial tissue, as it grows outward.

The second stage in tooth formation is that usually referred to as calcification. It is during this stage that apatite is laid down in the organic matrix which has already formed. Precipitation of the calcium phosphate begins at the outer tip of the enamel and proceeds in a direction opposite to matrix formation (3). Grenz-ray studies confirm the fact that calcification occurs in transverse relation to the layers formed by the matrix (7). Weinmann, Wessinger & Reed (8) showed that during calcification there occurs not only an influx

of mineral salts, but that there is also a removal of water and organic matter from the matrix. They suggested that proteolytic enzymes and phosphatases play important roles in calcification. Deakins (9) confirmed these observations and showed that the organic matter is removed first, and then water, in larger quantity. Actual hardening of enamel occurs when the water is removed. Deakins & Burt (10) found that calcium, phosphorus, and carbon dioxide separate in the matrix in a constant ratio during the entire period of calcification, indicating that a salt of fixed composition is being laid down.

The distinction between the two stages of enamel formation is very important. Wasserman (11) has pointed out that enamel hypoplasia may be due to incomplete formation of either the matrix or the calcified material. As Applebaum showed (7), defects in matrix formation may be concealed by subsequent calcification, but they are revealed when the tooth is decalcified. The second phase may be deficient due either to incomplete uptake of calcium, or to incomplete removal of water and organic matter. Since the latter process is a function of the ameloblast, which atrophies after enamel is formed, even a possible remineralization in later life will not restore the enamel to a normal condition, because organic matter can no longer be removed.

The theory of the mechanism of calcification in teeth is not as yet complete. There is a close relationship to calcification in bones, but the teeth offer advantages for studying the process, and much work has been done upon them.

The necessity of phosphatase for calcification has been recognized since the original publication of the Robison theory. Advances have now been made in locating the actual site of this enzyme in the calcifying tooth. Both Engel & Furuta (12) and Gomori (13) have shown that phosphatase does not occur in the ameloblasts or odontoblasts themselves, but is present in greatest amount at the point where calcium phosphate is actually precipitating. Gottlieb (14) pictures the ameloblasts as agents for concentrating and passing calcium salts to the site of calcification.

Further studies have done much to show the close connection between glycogen and calcification. Horowitz (15) showed that all areas in fetal heads in which calcification occurs contain both glycogen and phosphatase. The observation of Boyle & Wesson (16) that vitamin D helps calcification on diets low in calcium, if they are rich in carbohydrates but not if they are rich only in protein, is further evi-

dence of this relation. The evidence has been most convincingly presented, however, by Gutman & Gutman (17) and by Gutman, Warrick & Gutman (18). They showed that glycogen is necessary in the early stages of calcification to provide glucose-1-phosphate, which serves as a substrate for bone phosphatase. When the formation of the ester is prevented by phlorizin, calcification in calcifying cartilage stops. This theory implies that the glycogen-phosphorylase mechanism supplies the trigger which sets off further calcification by bone phosphatase. It also explains the nature of the Robison second mechanism.

It should be noted, however, that Roche & Mourgue (19, 20) believe that the function of phosphatase is merely to accumulate phosphate ions at the site of future calcification, where there is already independently fixed calcium. At a given time, this calcium is liberated from protein and rapid precipitation of calcium phosphate follows. This view fits in with the observed spread of the precipitate during calcification. Proteolytic enzymes must play a part in this process, and the removal of organic matter observed by Deakins (9) may be the trigger to start this calcification. Much of the work of Roche & Mourgue was done on the teeth of sharks, in which conditions may not be comparable to human teeth, but the calcification process seems to be very similar among most animals.

The studies of Kuyper (21, 22) have shown that precipitation of calcium phosphate depends to a great extent on the composition of the solution from which precipitation occurs. He suggests that material precipitated from a solution similar to blood serum has a composition resembling that of bone and dentin, while the precipitate in contact with a saliva-like solution has a composition similar to that of enamel. The presence of magnesium and citrate in the solution increases the solubility of the calcium salt.

FACTORS GOVERNING TOOTH FORMATION

Mineral metabolism.—The extreme sensitivity of developing teeth to changes in environment has long been known. However, Lund & Armstrong (23, 24) showed that the sensitivity of developing teeth to diets low in calcium was much less than that of bone, and even when bones were seriously affected, teeth were almost normal. Smith & Light (25) showed that acute calcium deficiency during development resulted in only mild hypoplasia in dog teeth. Apparently a simple calcium deficiency in the diet is not enough to produce

marked changes, probably because the bones supply enough calcium
to support calcification of the teeth.

The concentration of calcium is more important when fluorine is
added to the diet. Irving (26) and Lawrenz & Mitchell (27) showed
that a high calcium content in the diet depressed the sensitivity to the
deposit of fluorine in the teeth, and lowered the retention of fluorine
generally. Irving (28) demonstrated that when blood calcium was
low, the effects of fluorine were intensified. It appeared to alter dep-
osition of predentin in some way, so that later the calcification was
disturbed. Predentin deposited more than sixteen hours before ad-
ministration of fluoride was not affected in this way.

Effects in dentin at a high calcium/phosphorus ratio similar to
those produced by fluorine were also produced by injection of oxalate,
manganese, calcium, phosphorus, magnesium, molybdenum, and beryl-
lium (29). At low calcium/phosphorus ratios, the effects were not
so clear. None of these substances affected enamel. This suggests
that fluorine acts directly on enamel formation, but that its action
on dentin formation is indirect and depends on the calcium/phos-
phorus ratio.

The primary cause of fluorine hypoplasia of enamel is thus prob-
ably an interference with the action of the ameloblasts, and Grinstein
(30) and Hoffman, Schuck & Furuta (31) have shown that fluorine
actually causes degeneration of these cells. The changes are more
severe when a comparable amount of fluorine is given in the form
of 3-fluorotyrosine, rather than as sodium fluoride (32). The toxicity
of fluorine can be reduced by giving boric acid, aluminum sulfate, or
calcium hydroxide (33).

Evidence continues to accumulate that fluorine can be taken up
by the outer surface of enamel (34, 35), although deeper penetration
apparently does not occur after topical application to human teeth
(36). A possible mechanism for this fluorine uptake has been sug-
gested by Souder & Schoonover (37) who treated etched enamel with
sodium fluoride and then with a calcium sulfate solution to produce
a precipitate of calcium fluoride which filled the etched area almost
completely. Calcium salts in saliva may have a similar effect. Enamel
which has taken up fluorine is less soluble in acids than normal enamel
(35, 38), an effect also shown by the reduced etching of fluorosed
teeth by acid beverages (39).

The metabolism of other minerals is also of importance to the
developing tooth. A continuation of the work of Becks & Furuta

(40, 41) has shown that magnesium deficiency disturbs and eventually stops dentin formation, and causes abnormal calcification of pulp and cementum. Gagnon, Schour & Patras (42) noted a decreased rate of eruption.

Strontium severely decreases the formation of dentin (29, 43) but affects enamel less markedly, although in high concentrations it may cause localized areas of hypoplasia.

Manganese interrupts enamel formation in the apical fourth of the rat incisor (44), but probably produces most of its effect through its action on calcium and phosphorus metabolism (45). Boron has no effect on teeth (44). Cadmium causes a bleaching of the rat incisor analogous to that produced by fluorine (46, 47, 48). The effect is probably secondary, due to interference with iron metabolism, since the pigment of the incisor almost certainly contains iron, and both cadmium and fluorine produce anemia.

Vitamins.—The importance of vitamin intake for the proper development of the tooth has also been stressed. Burn, Orten & Smith (49) studied diets low in vitamin A for periods up to a year. In addition to the usual effects produced in short periods of such deficiency, they found a severe deformation in form of rat incisors, which were twisted into bizarre shapes, and the occurrence of supernumary teeth and tumors of the jaws. Bleaching of rat incisors in vitamin-A deficiency has been observed (50), again probably as a secondary effect of anemia. Glock and co-workers (3) found that a lack of vitamin A seemed to permit increased calcification, but a mixture of vitamins A and D_2 plus a little D_3 was not as effective as cod-liver oil in producing normal calcification. Irving (51) found that a combined A and D deficiency produced a marked stratification of the dentin which neither A nor D deficiency alone would cause. He attributed this to the action of a deficiency of A on a tooth already handicapped by a lack of D.

Irving (52) also noted that while addition of vitamin D or parathormone could improve calcification, they had no effect on calcification in predentin which had already formed. Thus, the proper maturation of dentin, so far as these substances were concerned, was determined at the time it was laid down. Ziskin *et al.* (53) demonstrated that doses of vitamin D (Ertron) of 60,000 to 100,000 U.S.P. units per kg. in rats or 300,000 units per day in humans showed no toxicity or tendency to produce pulp stones, but Becks (54) found that 10,000 U.S.P. units per kg. fed to dogs for 281 to 291 days produced pathological calcification of periodontal membranes, partial

degeneration of enamel epithelium, pulp stones, and calculus. Fish oils were less toxic than irradiated ergosterol. These findings were later confirmed (55) and it was shown that single massive doses of vitamin D produced the same defects as the chronic overdosage of the earlier studies (56).

Deficiencies of pantothenic acid in rats produced a number of changes in the soft tissues of the mouth and an anemia which was sometimes accompanied by depigmentation of incisor enamel. The presence of zinc increased these effects (57).

Kuether, Telford & Roe (58) found that in the absence of vitamin C, guinea-pig teeth showed abnormal calcification in the predentin and irregular calcification in the pulp. Odontoblasts showed degeneration, but ameloblasts were less seriously affected. The earliest signs of pathological change in dentin were noted when the blood level of ascorbic acid fell to 0.22 mg. per 100 ml., and when it fell to 0.08 mg. per 100 ml., severe damage resulted. Bourne (59) has indicated that vitamin C may be connected with calcification as well as with proper formation of matrix in dentin.

Much interest has recently centered on the depigmentation of rat incisors in vitamin-E deficiency. At first, results were confused, although Irving (60) described a specific atrophy of the enamel organ. Granados & Dam (61 to 64) have recently shown that depigmentation occurs in E deficiency only when unsaturated fatty acids are present in the diet. Depigmentation is more severe in maxillary than in mandibular incisors. These authors also have pointed out the relation between depigmentation and iron metabolism.

In a study of deficiency of vitamin K in which sulfadiazine was used to inhibit intestinal formation of the vitamin, Granados & Dam (65) found that lack of vitamin K had no effect on tooth formation, but sulfadiazine in all cases caused a decline in the rate of eruption and attrition of rat incisors.

Hormones.—There have been very few studies on the effect of hormones on the teeth during the last four years. However, Goldman (66) observed a distinctive injury to guinea-pig teeth due to hyperthyroidism, which he believed was due to changes in the odontoblasts. Vedani (67) showed that all unusual effects of fluorine on the teeth of hypophysectomized rats could be accounted for by the slower rate of growth of the tooth.

Effect of pathological disturbances.—It has long been known that hypoplastic changes in enamel and dentin may result from diseases

which occur when the tooth is forming. However, several workers have shown that such defects are not likely to result from the exanthematous diseases unless these are superimposed on a condition of nutritional deficiency (68, 69, 70). Enamel hypoplasia has been related to tuberculosis (71, 72) and to syphilis (73). Gersh & Restarski (74) exposed rats to artificial altitudes of 24,000 to 26,000 feet and found a disturbance of calcification which they attributed to anoxia.

STUDIES WITH RADIOACTIVE ELEMENTS

Radioactive strontium (75), sodium (76), and phosphorus (77) are adsorbed at 40°C. in the order bone > dentin > enamel by the powdered tissues, although in the case of phosphorus, this order is reversed at 64°C.

The small uptake of radioactive phosphorus by the intact tooth is now well established, and interest has turned to the exact portions of the tooth in which it is found. In normal teeth, the uptake is greatest in the dentin immediately surrounding the pulp (78, 79, 80). Crown dentin contains somewhat less and root dentin somewhat more (78, 80). The uptake of phosphorus is inversely proportional to the density of the tooth structure. Since enamel is denser than dentin, it is not surprising that uptake by enamel is only 20 to 35 per cent of that by crown dentin (80). In pulpless teeth, the uptake of phosphorus is less than in whole teeth, but still occurs, and is greatest in dentin near the cementum, which may indicate a nutritive function for that tissue (79, 81). Bodecker (82) has pointed out that the differences in permeability of various tooth areas to radioactive phosphorus under various conditions make it necessary to use caution in drawing conclusions when the past history of the tooth is unknown.

STUDIES ON CARIES

Experimental animals.—Possibilities for experimental study of caries have been increased by the discovery that several animals besides the rat can develop caries. Caries has been produced in hamsters (83 to 86) fed ground cornmeal instead of the coarse corn which is required to produce rat caries. The cotton rat develops caries on a diet high in sucrose (87 to 90), and rhesus monkeys are also susceptible (91). These studies are particularly interesting because of the varied methods for inducing caries, although fluorides in all cases

retard and carbohydrates increase the rate of development. These facts fit in with the suggestion of Cox (92) that initiation and progress of caries may proceed by two different mechanisms.

Dietary factors.—Boyd (93 to 97) has summarized his work which has extended over a number of years to show that caries can be sharply reduced by giving a diet markedly superior in all the essential nutritive elements. If the diet is excellent, large amounts of sugar do not increase caries. The effect is not due to a high fat content, nor to the fact that most of his subjects were diabetics. Howe, White & Elliot (98) have obtained similar results. It should be noted that Day (99, 100) has described a group of Indian children living on extremely deficient diets (almost devoid of sugar) who also showed almost no caries.

Boyd has not attempted to ascribe his results to any one factor in the diet, but several workers believe that under proper conditions, vitamin D can exert a protective action. McBeath & Verlin (101) found that addition of D to the diet of a group of children caused a reduction in caries. D_3 gave better protection than D_2. Brodsky, Schick & Vollmer (102) gave a single massive dose of A and D to children who had tuberculosis or had been exposed to it, and claimed a reduction in caries which lasted for a year. East (103, 104) and Dunning (105) made studies of large groups drawn from various geographical localities, and found that those individuals from regions with a large amount of sunshine showed less caries than those from less sunny regions. Again, the suggestion of a difference in mechanism of initiation and progress of caries may explain these effects, if it is assumed that vitamin D in some way retards the progress.

The claim that vitamin K inhibits acid production by oral bacteria (106) was confirmed, but it was shown that the effect was due to the general bacteriostatic action of quinones and was not specifically due to any vitamin activity (107).

Fluorides.—Considerable evidence has accumulated that topical application of sodium fluoride solutions to the teeth will reduce the incidence of caries (108, 109, 110). The mechanism of this action is still in some doubt. Bibby (111) believes that the primary mechanism is the reduction in acid solubility of fluorine-treated enamel. He has initiated a new and interesting study of the effect of various ions which may enter the apatite lattice of enamel as does fluorine and alter the solubility. He finds that lead salts, and especially lead fluoride, reduce enamel solubility to a degree greater than any other

compounds studied (112). The effect of this substance in clinical treatment remains to be determined.

The other possible mechanism is the reduction in bacterial activity by poisoning the phosphatases and other enzymes of the oral bacteria. Jay & Arnold (113) report that the lactobacillus count in individuals who have been exposed to fluorides is greatly reduced. The fact that iodoacetate will reduce the incidence of caries (114) also supports this mechanism, since it seems improbable that iodoacetate can reduce enamel solubility.

The remarkable powers attributed to the calcium and phosphorus balance in the soil of Deaf Smith County, Texas, for the reduction of caries (115) have been shown by McClure (116) to be nonexistent. The fluorides in the drinking water of this area account for the reduction.

Caries and proteolytic organisms.—A very significant theory of the mechanism of caries has recently arisen in opposition or addition to the classical Miller theory. It has been recognized that the protein matrix of the tooth must be destroyed in caries as well as the inorganic substance, and that proteolytic bacteria must therefore be present. In the past little importance has been ascribed to them.

In 1942, Hinds (117) suggested that bacteria might enter the lamellae or prism sheaths of enamel, which are protein in character, and Gottlieb & Hinds (118) pointed out that the yellow pigment usually found in a carious lesion was due to the action of proteolytic bacteria. This pigment is closely related to melanin (119). In this view, proteolytic bacteria are followed by acidogenic organisms which complete the work of destroying tooth structure (120). Confirmation of this theory has come from the work of Pincus (121) who has demonstrated the existence in the mouth of bacteria which attack protein anaerobically and from the very careful histological work of Frisbie, Nuckolls & Saunders (6) who have actually shown by microphotographs the bacterial invasion of a lamella. These bacteria send proteolytic enzymes ahead of themselves and clear the path to the dentino-enamel junction, where they spread to produce undermining caries, and penetrate into the dentin. Gottlieb (122) has recently pointed out that this type of caries can be identified by the yellow pigment which is produced, while the classical type of caries in which acidogenic bacteria lead the way may be detected by x-rays. This new hypothesis of the mechanism of caries promises to effect important theoretical and practical changes in work on caries in the future.

Other factors in caries.—The lack of relation between pregnancy and tooth decalcification or caries has again been emphasized (123, 124, 125).

A suggestion that there is a direct relation between the diastatic activity of saliva and caries (126) has been partially confirmed (127), and positively denied (128).

The observation that urea and alkyl dimethylbenzylammonium-chloride used in mouthwashes decreases caries incidence (129) has been related to the theory of Kesel, O'Donnell & Kirch (130) that free ammonia, which may arise from compounds like urea, or by deamination of free amino acids by mouth organisms, is a specific inhibitor of the growth of lactobacillus.

In conclusion, attention should be called to the techniques which have recently been worked out for the application of the electron microscope to the microstructure of the teeth (131, 132). Although no conclusions have yet been drawn, much may be expected from this method in the future.

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GROWTH FACTORS FOR MICROORGANISMS

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It is now ten years since Knight (1) and Lwoff (2) elaborated the view, foreshadowed by others, that the requirements of certain microorganisms for specific nutritional factors reflected a loss of their ability to synthesize these factors, which were looked upon as fundamental constituents of the protoplasm of a wide variety, if not of all, microorganisms. Knowledge of microbial nutrition was then fragmentary, but subsequent advances have firmly established the essential validity of this view and have extended it to include not only microorganisms but higher organisms as well. At present, microorganisms are known which require each of the water-soluble vitamins (with the possible exception of ascorbic acid) and each of the amino acids required by higher animals. On the other hand, no substances are yet known which are required for growth of microorganisms which do not also play some important biochemical role in the metabolism of higher plants or animals. The recognition of this fundamental similarity in the metabolic requirements of various organisms has facilitated greatly the identification of nutritional essentials of both microorganisms and animals (3, 4). Similarly, application of microorganisms to the quantitative determination of the vitamins (5) and the amino acids (6) which they require is rapidly extending knowledge of the distribution and importance of these substances in nature.

The recognition that the effectiveness of antibiotic agents may sometimes be due to their interaction with essential cellular metabolites, e.g., clavacin and sulfhydril groups (7), and that antibacterial agents can often be fashioned by varying the structure of known essential metabolites in a suitable manner (8 to 11) has served further to intensify interest in microbiological nutrition.

Knowledge of microbiological nutrition has thus assumed importance in many fields other than microbiology itself, and reviews dealing primarily with such related fields often deal extensively with studies on microbial nutrition (6, 9). This is well illustrated by a perusal of recent reviews in this volume dealing with the water-soluble vitamins. Very recently, two excellent and extensive reviews of growth factors and their relation to microorganisms have appeared

(5, 11) which cover most of the recent literature through 1944. In the present article, therefore, no attempt at an exhaustive treatment of the subject need be made. Emphasis will be placed instead upon more recent contributions to the general subject and upon those aspects of it which have been considerably developed or clarified during 1945.

GROWTH FACTORS OF KNOWN STRUCTURE AND RELATED COMPOUNDS

Biotin.—Studies on the activity of structures related to biotin continue. The methyl ester of the sulfoxide of biotin has the same activity for yeast as biotin itself, but is inactive for *L. casei* (12). Although data for the free sulfoxide have not been reported, it probably shows the same activity as its methyl ester, since treatment of natural products with hydrogen peroxide destroys all biotin activity for *L. casei,* but very little for yeast (13). More drastic oxidation procedures, such as treatment with potassium permanganate, oxidize biotin to its sulfone, which has about 0.1 per cent of the activity of biotin for yeast when compared at low concentration levels, but which does not permit at any concentration more than about one-third the growth obtained with optimal amounts of biotin (14). The product is inactive for yeast in the absence of aspartic acid. For *L. casei, L. arabinosus, Staph. aureus,* some streptococci and pneumococci (12, 14), and for the mold *N. crassa* (15), biotin sulfone is inhibitory; in all cases addition of extra biotin counteracts the inhibition. Growth of *E. coli,* which synthesizes its own biotin, was not inhibited by biotin sulfone (12); a mutant culture, which required added biotin for growth, was inhibited by this product (15).

Hydrogenolysis of biotin opens the tetrahydrothiophene ring and eliminates the sulfur atom (16). The activity of the resulting product, desthiobiotin, has been extensively investigated (15 to 20). Forty-five biotin-requiring organisms, including yeasts, molds, and bacteria, could be classed (17) in three groups with respect to their behavior toward desthiobiotin: (*a*) desthiobiotin fully replaced biotin for growth of many species of yeasts and molds, and for one bacterial species, *Leuconostoc mesenteroides;* (*b*) desthiobiotin did not replace biotin for several organisms (e.g., *L. casei, Ceratostomella pini*) but acted as an inhibitor; the inhibition could be overcome by additional biotin; and (*c*) desthiobiotin neither replaced biotin nor acted as an inhibitor for several organisms (e.g., *L. arabinosus* and *Rhizobium*

trifolii). Cultures which utilize desthiobiotin for growth convert it to a substance, presumably biotin, which replaces biotin for growth of organisms unable to utilize desthiobiotin (19, 20, 21). Excellent evidence that biosynthesis of biotin actually proceeds through desthiobiotin is provided by Tatum (15). Two mutant cultures of *Penicillium chrysogenum* were obtained which required biotin for growth. One of these, but not the other, could utilize desthiobiotin. The latter culture, when grown with minimal quantities of biotin, produced a substance corresponding to desthiobiotin in its growth-promoting properties for other organisms. The amount of this substance produced was increased almost twenty-fold by addition of pimelic acid, which is known to function as a precursor of biotin for the diphtheria organism (22). The synthesis of biotin by this culture apparently fails because of its inability to carry out the reaction: desthiobiotin → biotin; desthiobiotin therefore accumulates and biotin itself must be supplied for growth.

Several other inhibitory derivatives or analogues of biotin have been prepared. These include ε-imidazolidone caproic acid (12, 18) and a series of compounds in which the tetrahydrothiophene ring of biotin is replaced by a benzene or cyclohexane ring (23) and the length of the side chain is varied. Some of the latter compounds effectively inhibited yeast growth at a concentration of 3×10^{-7} moles per liter. In all cases sufficient amounts of biotin prevented inhibition.

Other derivatives or analogues of biotin which are effective in replacing biotin for some organisms include the diaminocarboxylic acid derived from biotin by opening the ureido ring (18), the "desthiodiaminocarboxylic acid" (ζ, η-diaminopelargonic acid) derived from this by removal of sulfur (18, 24) and oxybiotin (25) or O-heterobiotin (26), in which the ring sulfur of biotin has been replaced by oxygen. The first two products are about 10 per cent as effective as biotin in promoting yeast growth but are inactive for other organisms tested (18, 24). In contrast, *dl*-oxybiotin is highly active. Assuming only one isomer to be active, as is true for biotin (19), oxybiotin has the following activities (26 to 29) for various organisms (biotin = 100): *L. arabinosus*, 100; *L. casei*, 40 to 80; yeast (six strains) 40 to 50. The activity of a number of derivatives of oxybiotin has also been described (29). Preliminary indirect assay (28) indicated that yeast might convert oxybiotin to biotin during utilization, but development of a reliable method for the determination of both oxybiotin and biotin in mixtures of the two (30) showed that yeast

cells or cells of *Rhizobium trifolii* grown with oxybiotin contained only oxybiotin. Natural materials tested, such as yeast extract, contained only biotin, as did yeast cells grown with desthiobiotin (30).

Choline.—At present, pneumococci (31) and artificially produced mutant cultures of *Neurospora* (32) are the only microorganisms known to require external supplies of choline for growth. Of the naturally occurring compounds tested, only ethanolamine was able to support growth of the pneumococcus in the absence of choline (33). A large number of synthetic analogues supported growth of this organism, however, including dimethylethanolamine, monomethylethanolamine, triethylcholine, diethanolamine, and others. All such active compounds contained the N—C—C—OH or N—C—C—C—OH linkage. Examination of the active compounds indicated that the pneumoccus required choline primarily for lipid synthesis, rather than for transmethylation (33). Acetylcholine was inactive.

In contrast to the pneumococcus, two genetically different mutant strains of *Neurospora* were unable to utilize ethanolamine in place of choline. Acetylcholine, arsenocholine, phosphorylcholine, dimethylaminoethanol, monomethylaminoethanol, and methionine showed activity in varying degrees. Betaine, creatine, and a number of more distantly related compounds were inactive (34).

Hematin.—That hematin or the corresponding iron-free porphyrin, protoporphyrin, is essential for growth of several bacteria of the *Hemophilus* group (35) and for several parasitic protozoa (36) has been known for several years. In a preliminary study of the relationship of structure to the growth-promoting properties of various porphyrins for *Hemophilus influenzae*, it was found (37) that porphyrins which contained no vinyl groups, such as hematoporphyrin, mesoporphyrin, and coproporphyrin, would not support growth. The corresponding iron porphyrins sometimes did permit growth, e.g., iron mesoporphyrin. Although the organism grew well with iron mesoporphyrin, it did not reduce nitrate to nitrite under these conditions, as it did when grown with hematin or protoporphyrin. The latter reaction appears therefore to be incidental rather than essential to growth under ordinary conditions. Some of the iron-free porphyrins lacking vinyl groups inhibited the growth-promoting action of those compounds which supported growth, and the inhibition was competitive in nature. This was interpreted as evidence for the competition of the various porphyrins for the specific proteins of the heme enzymes.

Nicotinic acid, nicotinamide, and cozymase.—To the list of vita-

mins which stimulate yeast growth (38) must be added nicotinic acid, which is required for growth by a large number of lactose-fermenting yeasts (39, 40, 41).

When solutions containing asparagine and glutamic acid are heated, a substance is formed in minute amounts which replaces nicotinic acid for a variety of microorganisms (42). This was isolated and identified as nicotinamide (43). Together with findings to be cited later in connection with vitamin B_6, this result emphasizes that media should not be considered as qualitatively or quantitatively identical before and after heat sterilization.

Where differences of availability of nicotinic acid and nicotinamide occur, nicotinamide has usually been found most active; some organisms such as certain *Pasteurellae* require nicotinamide, and cannot utilize nicotinic acid (44). Inasmuch as some organisms (e.g., *Hemophilus parainfluenzae*) can utilize neither nicotinic acid nor nicotinamide, but require coenzyme I or II, these facts are sometimes interpreted to mean that the synthesis of the coenzymes occurs via the stages: nicotinic acid $\rightarrow$ nicotinamide $\rightarrow$ coenzyme I or II (11). This picture is probably an oversimplification, however, for a strain of *Leuconostoc mesenteroides* has been found which requires nicotinic acid for growth, and is unable to utilize nicotinamide (45). For the diphtheria organism, too, nicotinic acid is approximately ten times as effective as nicotinamide in promoting growth (46). These results recall the finding (47) that the V-factor content of human erythrocytes is increased by ingestion of nicotinic acid but not of nicotinamide.

The specificity of compounds structurally related to nicotinic acid in stimulating or inhibiting growth of microorganisms has been summarized elsewhere (11). It should be added that N-methylnicotinamide (48) and nipecotic acid (49) do not support growth of *L. arabinosus*. A combined form of nicotinic acid occurs in cereal grains which shows full activity for *L. arabinosus* only after hydrolysis. From the chemical behavior and growth-promoting properties of crude concentrates, it was concluded to be an ester of nicotinic acid with an unidentified, acidic and water-soluble compound (48). In contrast to results with mice (50), no specific inhibition of growth of microorganisms which could be prevented by nicotinic acid was produced by 3-acetylpyridine (50, 51).

Nicotinamide riboside satisfies the requirement of *Hemophilus parainfluenzae* and *H. influenzae* for V-factor, but somewhat less efficiently than diphosphopyridine nucleotide (DPN). Growth response

to increasing concentrations of the nucleoside follows a curve which differs from that obtained with intact DPN. Triphosphopyridine nucleotide (TPN) is even less efficiently utilized than is nicotinamide riboside, and the growth curve obtained with it parallels closely that obtained with the nucleoside, indicating that TPN may be broken down to the riboside, then resynthesized to DPN by these organisms. Desamino DPN is also effective as V-factor (52).

Pantothenic acid.—Examination (53) of the response of thirty-three strains of lactic acid bacteria to pantothenic acid has confirmed previous reports (54, 55, 56) to the effect that all strains of these organisms require pantothenic acid for growth. In no case were the lactone and β-alanine moieties of pantothenic acid able to substitute for the intact molecule. At present, the diphtheria bacillus, *Rhizobium trifolii, Brucella suis,* and yeasts are the only organisms known to respond to β-alanine. A study (57) of sixteen strains of *Saccharomyces cerevisiae* and one of *S. carlsbergensis* revealed only a single strain that was unable to utilize β-alanine in place of pantothenic acid. Several of the strains did not grow as heavily with excess β-alanine as with excess pantothenic acid. In no case did simultaneous addition of the lactone portion of the pantothenic acid molecule improve the response to β-alanine.

The requirement of *Acetobacter suboxydans* for pantothenic acid is satisfied by the lactone moiety of the vitamin (58), which appeared more active than pantothenic acid in supporting growth of this organism on the earlier media used. The amounts of pantothenic acid necessary for growth on these media (1 to 5 µg. per 10 cc.) were, however, considerably greater than those required for other organisms on optimal media. In a subsequent study (59), a medium was developed in which the requirement of the organism for pantothenic acid (0.1 to 0.5 µg. per 10 cc.) was more in line with that observed for other microorganisms. On this medium the lactone moiety of pantothenic acid was only 20 to 25 per cent as effective as an equimolar amount of pantothenic acid. Hydrolysis of the lactone increased its activity about five-fold, to that of pantothenic acid. Addition of β-alanine improved the response to small amounts of the hydrolyzed lactone. The medium and organism can be used for the quantitative bioassay of the non-nitrogenous portion of pantothenic acid in hydrolysates of natural materials (59).

A strain of *Clostridium septicum* is also able to utilize pantolactone or pantoic acid in place of pantothenic acid, although somewhat less

efficiently; presence of β-alanine does not increase response to the lactone. Assays of cells after growth showed that the lactone had not been utilized directly, but had been used to synthesize pantothenic acid (59a).

The inhibitory properties of pantoyltaurine for microorganisms which require pantothenic acid are well known (60, 61, 62). A large number of other analogues of pantothenic acid, in which the structure of the β-alanine portion of the molecule has been altered, have been prepared. These are listed in Table I.

TABLE I

INHIBITORY ANALOGUES OF PANTOTHENIC ACID (RCONHCH₂CH₂COOH)

Compound	Reference	Compound	Reference
RCONHCH₂CH₂SH (RCONHCH₂CH₂)₂S₂ (RCONHCH₂CH₂)₂S (RCONHCH₂CH₂)₂SO (RCONHCH₂CH₂)₂SO₂	 63	RCONHCH₂CH(CH₃)₂ RCONHCH(CH₃)CH₂CH₃ RCONHC(CH₃)₂CH₃ RCONHCH₂CH₂CH(CH₃)₂ RCONHC₂(CH₂)₅CH₃ RCONHCH₂CH₂C₆H₅ RCONHCH₂CH₂OCH₃	67
RCONHCH₂CH₂CH₂OH* 64, 65			
RCONHCH₂CH₂CHOHCH₃* 66		RCONHCH₂CH₂COC₆H₅ 68	
RCONHCH₂CH₂OH* RCONHCH₂CH₃ RCONHCH₂CH₂CH₃ RCONHCH₂CH=CH₂	 65	RCONHCH₂CH₂CN RCONHCH₂CH₂CH₂NH₂	... 67
		RCONHCH₂CH(CH₃)COOH* 66, 69	
RCONHCH(CH₃)₂ RCONHCH₂CH₃CH₂CH₃	... 67	RCONHCH(CH₃)CH₂COOH* RCONHCH₂CHOHCOOH*	66

* Not obtained as analytically pure compounds.

Inhibition by these compounds is prevented in every case by the addition of sufficient quantities of pantothenic acid; the relationship is competitive in nature. In contrast to the sulfonamides, where variations in structure affect the inhibitory action of the compound in the same manner toward almost all organisms (when tested *in vitro*), the relative inhibitory action of the pantothenic acid analogues for a given organism cannot be predicted from a knowledge of their relative activities for another organism (65, 66, 67).

At low concentrations, salicylate exerts a specific inhibitory effect on growth of *S. aureus, E. coli,* and some other organisms, which is counteracted by addition of pantothenic acid (70). The relationship of the two substances is not a competitive one; it is believed that the

salicylate specifically prevents synthesis of pantothenic acid. This interpretation is supported by the fact that this specific type of inhibition is not observed with organisms such as *Proteus morganii,* which require added pantothenic acid for growth. An apparently similar inhibition of *E. coli* by mandelic acid is likewise counteracted by added pantothenic acid (71). That inhibition by such substances differs fundamentally from that produced by structural analogues of pantothenic acid is apparent, since the former phenomenon is observed most characteristically with organisms which synthesize pantothenic acid, while the latter group of compounds is characteristically inhibitory only for those organisms which do not synthesize pantothenic acid.

No extended studies of the effect of compounds related in structure to β-alanine on the utilization of this compound have appeared. In most cases taurine is without effect on the utilization of β-alanine (57, 60). α- and β-substituted β-alanines such as isoserine or β-aminobutyric acid inhibit growth stimulated by β-alanine in a competitive fashion (72). During an attempt to utilize the response of yeast to β-alanine for the quantitative determination of this substance in natural materials, a variety of nitrogenous materials (α-amino acids, peptides, asparagine, etc.) was found to inhibit utilization of small amounts of β-alanine. The same substances had little or no effect on the utilization of pantothenic acid (57).

Riboflavin.—The requirement for riboflavin of a large number of microorganisms belonging to various groups of lactic acid bacteria has been determined (55, 56, 73, 74). Requirement for this factor is distributed somewhat at random; different strains of a single species may differ completely as regards their behavior toward it. A more or less analogous situation occurs with many of the other vitamins, and as a general rule the nutritive requirements determined for a single strain do not necessarily represent those of the species as a whole. At present, no yeasts or lower fungi are known which require added riboflavin for growth. Such organisms synthesize riboflavin, however, and the production by artificial means of a mutant of *Neurospora* which requires riboflavin under some conditions (75) indicates that such organisms may occur naturally.

An analogue of riboflavin in which the two methyl groups are replaced by chlorine (6,7-dichloro-9-*d*-ribitylisoalloxazine) inhibits growth of *Streptobacterium plantarum;* the inhibition is prevented by addition of riboflavin (76). From the observation that the ratio of analogue to riboflavin required for inhibition increased with time of

incubation, it was concluded that the organism slowly synthesized riboflavin. However, an analogous phenomenon occurs with certain analogues of pantothenic acid and *L. casei,* which does not synthesize pantothenic acid (67). A more probable explanation lies in the difficulty of differentiating by the comparatively crude measurements made "complete inhibition" from a greatly increased generation time of the test organism. The effect of isoriboflavin (77) and galactoflavin (78) on growth of microorganisms has not been reported. These compounds have antiriboflavin activity in the rat. A phenazine analogue of riboflavin (2,4-diamino-6,7-dimethyl-9-ribityl-9,10-dihydrophenazine) has antiriboflavin activity for *L. casei* (79). The growth-promoting activity of mono-, di-, tri-, and tetrasuccinic acid esters of riboflavin for *L. casei* paralleled that found for rats, but was somewhat lower in each case (80).

Thiamine.—The very extensive literature dealing with the relationship of this widely required growth factor to microorganisms has been fully summarized elsewhere (5, 11). The wide variability in the availability of thiamine pyrophosphate merits some comment. This compound is almost inactive in supporting growth of yeast under conditions where thiamine is highly active (81). For *Streptococcus salivarius* (82) and *Lactobacillus fermenti* (83), it is 1.3 to 1.5 times as active on the molar basis as thiamine. Finally, there are exacting strains of the gonococcus which require cocarboxylase for growth and are unable to utilize thiamine (84). Yeast is known to convert thiamine to cocarboxylase; the inactivity of the latter as a growth factor for yeast probably means that the living yeast cell is impermeable to the pyrophosphate. This is obviously not true for the cells of many other microorganisms.

Vitamin B_6—pyridoxal, pyridoxamine, and pyridoxine.—The first indication that vitamin B_6 is multiple in nature was the observation in 1942 that the "pyridoxine" content of natural materials, as measured by available methods, was entirely insufficient to account for their growth-promoting activity for certain lactic acid bacteria (85) in vitamin B_6-free media if pyridoxine was the only active compound present. One or more substances having heightened activity for these organisms were formed from pyridoxine when the latter was fed to rats or men. This substance was present in the tissues of animals and the amount present varied with the pyridoxine content of the ration. Although pyridoxine was apparently required for growth of *Streptococcus fecalis* R (*S. lactis* R), it was not absorbed from the medium

to any measurable extent even when present in concentrations which limited growth. All of these facts pointed to the formation from pyridoxine of a more active substance (for *S. fecalis*), which was the form of the vitamin necessary for utilization. To differentiate it from pyridoxine, this substance was provisionally called "pseudopyridoxine" (85). It was subsequently found that some of this substance was formed from pyridoxine when the latter was autoclaved with bacteriological media or with amino acids (86). Detailed investigation of the effects of various chemical treatments on pyridoxine showed that two different substances having greatly heightened activity for lactic acid bacteria existed; an amine, formed by amination procedures, and an aldehyde, formed by oxidative procedures (87, 88). Synthesis (89, 90) of various proposed structures (88) for these compounds, and subsequent microbiological tests on the synthetic compounds (87, 91) established 2-methyl-3-hydroxy-4-aminomethyl-5-hydroxymethyl-pyridine as the active amine, and 2-methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine as the active aldehyde. These were named *pyridoxamine* and *pyridoxal,* respectively (87). Investigation (91) of the activity of these compounds for a variety of microorganisms previously known to require vitamin B₆ for growth revealed responses of the following types: (*a*) organisms for which pyridoxine and pyridoxamine were comparatively inactive and for which pyridoxal was highly active, e.g., *Lactobacillus casei;* (*b*) organisms for which pyridoxamine and pyridoxal had high activity but for which pyridoxine was almost inactive, e.g., *Streptococcus fecalis, S. lactis,* and *S. zymogenes;* and (*c*) organisms for which each of the three compounds had essentially equal activity, e.g., *Saccharomyces carlsbergensis, Mycoderma valida, Ceratostomella ulmi,* and rats. For two yeasts pyridoxamine and pyridoxal were slightly less active than was pyridoxine. If it is agreed that vitamin B₆ plays a similar role in all organisms, the above facts can be rationalized from the following reactions:

$$\text{pyridoxine} \rightleftharpoons \text{pyridoxal} \rightleftharpoons \text{pyridoxamine}$$
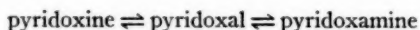
Organisms of group (*a*) above are unable to carry out the indicated conversions readily; organisms of group (*b*) readily interconvert pyridoxal and pyridoxamine, but not pyridoxine and pyridoxal, while all of the conversions are readily made by organisms of group (*c*). This concept gives to pyridoxal the central position in the vitamin B₆ group, a position which is also indicated from its role as precursor of

pyridoxal phosphate (92), which functions as coenzyme for amino acid decarboxylation (92, 93, 94) and for transamination (95, 96).

The natural occurrence of pyridoxal and pyridoxamine in natural materials has been demonstrated (97). In acid-hydrolyzed extracts these two compounds appear to account almost wholly for the "pseudo-pyridoxine" activity, and constitute the major portion of the total vitamin B_6 present in many metabolically active tissues (97). Recent evidence obtained largely with microorganisms (98) indicates that in unhydrolyzed tissue extracts (or in those hydrolyzed by mild procedures) and in sweat (99) and urine (100), additional substances which have vitamin B_6 activity for some organisms may be present. At least one of these is destroyed by acid treatment (98).

The existence of these new forms of vitamin B_6 requires a re-appraisal of much of the older literature dealing with vitamin B_6. For example, the fact that progressively smaller amounts of pyridoxine were required for growth of L. casei as the oxygen tension was increased (101) could be interpreted as reflecting a greater requirement for pyridoxine for growth under anaerobic conditions. A more valid explanation appears to be that formation of pyridoxal, which is much more active for this organism than pyridoxine, is promoted under oxidizing conditions.

It is clear that none of the organisms proposed for assay of vitamin B_6 responds specifically to pyridoxine, as was claimed (102, 103, 104) before the nature of "pseudopyridoxine" was partially elucidated. For determination of total vitamin B_6 those organisms which respond equally to the three known forms of the vitamin, such as S. carlsbergensis or N. sitophila, are preferable (91). The differential microbiological determination of the three known forms of vitamin B_6 (97) which was developed and used during work on the chemical nature of "pseudopyridoxine" has been applied to natural materials only in conjunction with various chemical procedures to establish the presence of pyridoxamine and pyridoxal in such materials. In its present form the procedure is unreliable without such supporting data.

The activity of several compounds related to pyridoxamine and pyridoxal in promoting growth of different representative microorganisms has been determined (91). The isomers of pyridoxamine and pyridoxal in which the groups occupying positions 4 and 5 of the pyridine ring have been interchanged have negligible activity for lactic acid bacteria; yeast utilizes the isomeric aldehyde with reduced efficiency. The lactone of α-pyracin (105) is inactive; that of β-pyracin

has a very slight but entirely negligible activity for all organisms tested. The latter two products have been reported to stimulate growth of *L. casei* (105, 106) and chicks, but this activity is observed under an entirely different set of conditions, and should be distinguished clearly from their lack of activity as vitamin B_6. For *S. fecalis* R (107) and a number of other enterococci (56), excess alanine completely replaces vitamin B_6. With *L. casei,* alanine is ineffective alone, but replaces vitamin B_6 in the presence of an unidentified supplementary factor (108). Surprisingly, $d(-)$-alanine is more active in this respect for *S. fecalis* than is $l(+)$-alanine, while only the former isomer can be used effectively by *L. casei* (108). Structural relationships between alanine and vitamin B_6 suggest (107) that alanine might serve as a precursor in the synthesis of vitamin B_6, but the observation that detectable quantities of codecarboxylase are not synthesized when *S. fecalis* is grown with alanine in place of vitamin B_6 argues against this hypothesis (96, 109).

Purines and pyrimidines.—A number of interesting reports in addition to those summarized elsewhere (5, 11) have appeared. Several strains of Group A hemolytic streptococci grew on a semi-synthetic medium containing the known vitamins only when serum from certain animals was added. The serum could be replaced by a number of pure compounds including xanthine, guanine, hypoxanthine, guanosine, adenosine, and others (110). Adenine was inactive, although it is known to promote growth of other similar organisms (111). Many cultures of these organisms did not require these substances; those which did were distributed at random among almost all of the serological types. While the pyrimidine nucleosides are from 10 to 60 times as active as the free pyrimidines in supporting growth of certain mutant cultures of *Neurospora* (112), the purine nucleosides and nucleotides are no more active on a molar basis than the free purine bases for a mutant culture requiring adenine, hypoxanthine, or oxyadenine (113). Purine and pyrimidine bases are also required for a protozoan organism, *Tetrahymena geleii* (114).

Analogues of guanine, adenine, and hypoxanthine in which carbon number 8 of the purine nucleus was replaced by nitrogen to yield triazole pyrimidine derivatives (115) have been prepared. These substances inhibit growth of *E. coli* and *S. aureus;* the inhibition is specifically prevented by the corresponding purine bases. The antibacterial indices ranged from 35 to 6400 for these compounds. The guanine analogue acted synergistically with the sulfonamides in inhib-

iting growth of both organisms (115). Growth of *E. coli* and *L. casei* is similarly inhibited by thiouracil; this effect is prevented by uracil but not by thymine or purine bases (116). The corresponding analogue of thymine was without effect on growth of *E. coli* but inhibited growth of *L. casei* when the latter was grown with thymine in place of folic acid.

GROWTH FACTORS OF UNKNOWN CONSTITUTION

Folic acid, L. casei factor, vitamin B$_c$, etc.—The synthesis of one form of this important growth factor has been announced (117), but no details are available. Experimental use of the synthetic material will undoubtedly dispel much of the confusion which now permeates this field; detailed discussion of the subject should therefore be deferred until later. It is already clear, however, that the multiple forms of the vitamin which have been isolated and which differ in their activity toward the two test organisms, *S. fecalis* R and *L. casei*, are each convertible under proper conditions to a substance having very high activity for both of these microorganisms and for animals. Digestion with a widely distributed enzyme from animal tissues accomplishes this for the vitamin B$_c$ conjugate (relatively inactive for both *L. casei* and *S. fecalis*) from yeast (118) and for one form of the crystalline *L. casei* factor (high activity for *L. casei*, relatively little activity for *S. fecalis*) (119); judged solely on the basis of activity the resultant product appears in each case to be identical with the vitamin B$_c$ from liver (equally active for *L. casei* and *S. fecalis*). The SLR factor (highly active for *S. fecalis*, comparatively inactive for *L. casei*) is converted to a similar substance during growth of microorganisms which utilize it (120), and by resting cell suspensions of various enterococci (121). These and other data indicate that a single "master compound" occurs naturally in a variety of combined or modified forms.

The list of microorganisms known to require this substance is not extensive. Requirement for it is distributed at random among the enterococci—seven of nineteen strains examined required it (56)— while none of thirty-six strains of *S. lactis* required it (55). Several, but by no means all, lactobacilli require it, as does *Clostridium tetani* [see (5)]. It is also involved in the nutrition of protozoa [*Tetrahymena geleii* (114)] but has not yet been identified as a nutritional requirement of yeasts or other fungi.

The observation that thymine replaces folic acid for growth of

S. fecalis (122) and *L. casei* (123) continues to elicit interest. While replacement is complete for *S. fecalis, L. casei* does not grow as well with excess thymine as with excess folic acid (124, 125). Thymidine is as active as thymine; the concentration of thymine necessary for adequate growth is about 5000 times higher than that of folic acid (124). The mechanism by which thymine produces its effect is unknown. Cells grown with thymine in place of folic acid do not contain detectable quantities of folic acid; it is possible, therefore, that the cells do not synthesize folic acid but grow in its absence when thymine is present. For such growth, other purine bases are also required; it is possible that folic acid is concerned primarily in the mechanism by which thymine and purine bases are synthesized by these lactic acid bacteria and when these substances are supplied in excess, folic acid is no longer required (124). However, the complete extraction of folic acid from natural materials presents many unsolved difficulties (121), and failure to detect it in extracts of bacterial cells is not at present conclusive evidence for its absence from such cells.

5-Methylcytosine, 5-methylisocytosine, 5-methyl-2,4-diaminopyrimidine, and 5-methyl-2-oxy-4-thiopyrimidine all are active in place of thymine, but only the thio-compound is as active. No pyrimidine lacking the 5-methyl group has any activity, while some, such as 5-hydroxy or 5-aminouracil, which are configurationally closely related to thymine but lack the methyl group, inhibit growth. This inhibition is generally overcome by additional thymine or folic acid (126).

Reports of chloroform-soluble factors from liver which replace folic acid for *L. casei* and *S. fecalis* (127) and which must differ markedly from folic acid (128) cannot be accurately evaluated in the absence of more complete data. The effect may be related to that of α- and β-pyracin, which appear to increase the response of *L. casei* to certain naturally-occurring combinations of folic acid (105, 129).

Factors present in enzymatic protein digests.—Several workers have reported substances which are either essential for growth, or greatly stimulate the rate of growth of hemolytic streptococci (130, 131), lactic streptococci (132, 133), and lactobacilli (131, 134) when grown on media containing the known essential amino acids, vitamins, and mineral elements. Evidence for the identity of several of these factors has been presented, and the active substance called "strepogenin" (131). The substance (or substances) occurs richly in certain types of peptone (134), in tryptic digests of purified casein (133,

135), and other purified proteins (135). It is destroyed by acid hydrolysis and is adsorbed by activated charcoal with difficulty (131, 133, 134, 135). Much evidence points to a relationship of this factor to glutamine and asparagine. Thus the latter two substances, either alone (133, 134) or admixed with *p*-aminobenzoic acid and pyridoxal (136), duplicate the effect of enzymatic protein digests for several lactic streptococci and for *L. casei*. Glutamine is most effective in this regard, but the active substance cannot be glutamine, since it is stable to autoclaving (134, 135), and since enzymatic digests of casein are much more active than can be accounted for on the basis of their glutamic (or aspartic) acid content (133). Glutamine or asparagine may suffice as growth factors for certain organisms, but may be involved in the synthesis of more highly active compounds which are found in enzymatic protein digests (133). However, glutamine appears to stimulate growth of some microorganisms on media containing ample quantities of peptones (134), and peptides of glutamine so far tested have been ineffective in replacing glutamine for hemolytic streptococci which require the latter substance (137). Although suggestive, the evidence for a relationship to glutamine is thus not conclusive. Of the proteins tested, crystalline insulin yields the most active digests, which are somewhat more effective in promoting growth of *L. casei* than the best concentrates of the factor previously reported. The active substance in such concentrates appears to be a peptide (135).

An unrelated substance, also present in enzymatic digests of proteins and probably of peptide nature, permits growth of *L. casei* in the absence of vitamin B_6 when *dl*-alanine is also present in the medium (108).

Other unidentified growth factors.—To the unidentified bacterial growth factors listed in a recent review (5) should be added a substance required for growth of *Clostridium perfringens* (138). A concentrate purified about 200-fold over the starting material was stable to acid and alkali at room temperature, but was inactivated at 100°. It was destroyed by nitrous acid, but was stable to permanganate at pH 7.5; it could not be replaced by any known growth-factors. Treatment with anhydrous hydrochloric acid in ethanol inactivated the product, but activity was restored by mild hydrolysis. Existence of an alkali-labile substance in yeast extract necessary for growth of *Lactobacillus gayoni* and not replaceable by any known vitamins has also been indicated (139).

MISCELLANEOUS

Nutrition of protozoa.—Progress in this difficult field remains slow. The nutritional requirements of different organisms of the group vary to an even greater extent than among bacteria. To the previously known substances—thiamine, hematin, cholesterol, and ascorbic acid (140)—required by certain of these organisms have been added pyrimidine and purine bases (114), amino acids (141), riboflavin, niacine, pantothenic acid, and vitamin B_6 (142). Several poorly characterized heat stable and heat labile substances of unknown nature are required by various organisms investigated (114, 142 to 145). One of these appears similar to "strepogenin" in nature (114). Two heat labile factors, which occur in a plasmoptyzate of *Pseudomonas fluorescens* and in unheated, pressed yeast-juice and permit growth of *Colpoda duodenaria* and *Paramecium multinucleata* respectively, appear to be proteins (142, 145).

Nutritional mutants of microorganisms.—The production by ultraviolet or x-ray irradiation of mutant cultures of *Neurospora* which differ from parent cultures in their inability to synthesize factors essential for their growth is an established technique, and has been reviewed elsewhere (75, 146, 147). Application of similar techniques to *E. coli* resulted in a twenty-fold increase in the rate of mutation of irradiated over non-irradiated cultures (148). Cultures of *E. coli* which required biotin and threonine were produced (149); subsequent irradiation of the biotin-requiring culture produced several additional mutants, each of which required biotin plus some additional growth factor. Such additional requirements were thiamine or its constituents, and several amino acids. Besides mutants requiring known vitamins and amino acids, three cultures were produced which grew only when yeast extract was added to the culture medium (148). Similar irradiation of *Acetobacter melanogenum* also resulted in production of stable strains requiring either glycine, serine, leucine, or adenine (149). These results suggest that biosyntheses in bacteria, as in *Neurospora* and higher organisms (147), are controlled by specific genes.

When cultures of *E. coli* are subjected to one or more of three viruses active against this organism, the overwhelming majority of the cells are destroyed. A few mutants develop, however, which are resistant to action of the viruses. Twenty-eight of fifty-seven mutant cultures thus produced failed to grow on a minimal medium which

supported growth of the parent strain, or upon this same medium supplemented with known vitamins and amino acids. Growth was obtained, however, upon addition of minute amounts of yeast extract, indicating an induced requirement for a supplemental factor of unknown nature (150).

Synthesis of growth factors by microorganisms.—The synthesis of growth-factors by organisms not requiring them is well recognized (5). Of greater interest are occasional reports of organisms which ordinarily require a vitamin, but which under special conditions grow in its absence without synthesizing detectable quantities of it. Reported examples of this phenomenon include growth of *S. fecalis* in the absence of vitamin B_6 when alanine is supplied (108, 109), growth in the absence of folic acid when thymine is supplied (124), and growth of *L. casei* in the absence of biotin when sufficient oleic acid or rice oil is present (151). Such cases merit further investigation. Adaptation to growth in the absence of previously essential metabolites by development of the power to synthesize them is much more common, and has been observed frequently (152, 153).

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PHOTOSYNTHESIS

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Most of the literature on photosynthesis up to the last year has been covered in an excellent book by Rabinowitch (1). Many important experimental and theoretical papers have, therefore, been omitted from the present article, in which an attempt will be made to discuss primarily selected recent experimental results which have a direct bearing on the fundamental nature of the process. The new papers in the field, both theoretical and experimental, generally consider photosynthesis to consist of many separate steps rather than being a simple integrated process for which a workable mechanism can be derived from a few kinetic measurements. The separate steps are often distinguishable experimentally; as parts of the over-all reaction, they are being investigated with the idea that, except for the photochemical process, the steps themselves may be similar in their biochemical nature to other life processes whose characteristics have been studied in more detail.

Now that photosynthesis *in vitro* is coming somewhat closer to realization even the most elaborate and ingenious theories based on deductions from kinetic data may before long be more easily susceptible to direct experimental proof by actual chemical identification of the still hypothetical intermediate products and reactants. The introduction to the review of blood coagulation by Chargaff (2) might equally well have been written as a preface to an article on photosynthesis in its present state.

Books and Reviews

Rabinowitch has correlated and critically interpreted the information available early in 1945 in a remarkably clear and complete treatise on photosynthesis. Its appearance is the outstanding recent event in the field. This book, written from the point of view of the physical chemist, presents a lucid account of the early discoveries as an introduction, then discusses the chemistry of photosynthesis and related processes in living organisms as well as of analogous inorganic reactions such as the photodecomposition of water and the reduction of carbon dioxide. Photosynthesis and chemosynthesis in bacteria are

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discussed with particular attention to the energy changes involved. The photoreductions and other reactions with hydrogen gas found in certain strains of anaerobically adapted algae are described. Many of the existing theories of photosynthesis are presented in a logical order and in a uniform terminology, thus making many of them comprehensible to this reviewer for the first time. The energy changes associated with the hypothetical partial reactions of photosynthesis are calculated and used in critically evaluating the various theories. Throughout the book there is an abundance of specific information of biological as well as of a physicochemical nature. The influence of various poisons and stimulants on photosynthesis is discussed in detail. Part II of the first volume deals with the structure and pigments of the chloroplasts including the photochemical behavior of the photosynthetic pigments in reactions other than assimilation. Even the more purely biological aspects of the subject are dealt with in a clear and logical style that is indeed rare in biological literature. The second volume of this work dealing with the spectroscopy and fluorescence of the pigments and with the kinetics of photosynthesis is scheduled for publication in 1946.

Previous general reviews of photosynthesis have been published within recent years (3 to 10). A general discussion of the role of photosynthesis in nature and also comments on suggested mechanisms such as phosphorylation theories has been provided by Gaffron (11). Carbon dioxide fixation, oxygen liberation, and the possible role of phosphate esters in photosynthesis are reviewed by Lardy & Elvehjem (12). The hydrogen metabolism of anaerobically adapted algae has been summarized by Gaffron (13).

QUANTUM YIELDS

The quantum yield of photosynthesis in *Chlorella* as determined by the Wisconsin group and confirmed by Emerson & Lewis (14), which has already been discussed (8), remains the most probable and the generally accepted value, 0.1 ± 0.02 molecules of oxygen produced per quantum of light absorbed. This has been confirmed by unpublished experiments of Rieke which have been done with great care. In a theoretical paper, however, Dorrestein, Wassink & Katz (15) speaking of the quantum yield work of Emerson referred to in the review of Franck & Gaffron (6) state:

These authors appear to reject Eichoff's [16] results, which we, however, should not like to ignore, the more so since some incidental observations of our

group have yielded efficiencies of 1:5 to 1:9 [0.25 to 0.11 molecules per quantum] in *Chlorella* even without severe precautions being taken as to obtain the most active cells. Later on [sic] we have been able to confirm these results, and found that with *Chlorella* in Warburg-buffer No. 9, at 29°, at least efficiencies of about 1:6 [0.17] are obtainable in experiments of prolonged duration, in sodium light intensities which allow definitely positive readings.

Tonnelat (17) reports an energy efficiency of 30 per cent (quantum yield 0.14) measured with an adiabatic calorimeter in green light. This value is also reasonably close to the unpublished results of Arnold and of the Wisconsin group (18) who obtained about 20 per cent energy efficiency [misquoted in (17) as 5 per cent] with red light which corresponds to a quantum yield of 0.093.

Rieke in unpublished work has found identical quantum yields for oxygen production in *Scenedesmus* and for hydrogen reduction by the same anaerobically adapted algae.

Emerson & Lewis (19) have carried their previous investigations further to make an elegant study of the variation of the quantum yield with wave length in *Chlorella* as a supplement to their similar experiments with the blue-green alga *Chroococcus* (20). The yield found for *Chlorella* photosynthesis, using total absorption of the light, remains at a constant level between 580 and 640 mμ and again reaches the same value at 690 mμ. There is some evidence, which the authors themselves do not consider certain proof, that the yield may, in the region of 660 ± 15 mμ, drop about 5 per cent below the maximum. Before this effect is established without doubt a more detailed discussion of the monochromator performance and particularly of the filters used for the elimination of stray light in the different wave-length regions compared would be highly relevant. The grating monochromator used in these researches has not been described in detail but it certainly is far superior with regard to the purity and intensity of the light to any other instrument used for similar experiments. It seems reasonable to accept as a real effect this small decline of quantum yield in the region of the spectrum which is absorbed more by chlorophyll-*b* than by chlorophyll-*a*. The explanation offered by these authors is that the light of wave length near 660 mμ is partly absorbed by chlorophyll-*a* but more so by chlorophyll-*b*. This light is, however, presumed to be of slightly too long a wave length for its efficient utilization by chlorophyll-*b* since it is on the long wave side of the chlorophyll-*b* fluorescence maximum *in vivo*. This hypothesis is plausible in view of the similar rapid decline in quantum yield which they found

just beyond the absorption peak of chlorophyll-*a* in the live cells. In this region near 720 mμ the cells still absorb appreciably but the photosynthetic yield is less than half of that prevailing near the peak of the red absorption band. Light of wave lengths longer than about 700 mμ is presumed to be of too small an energy content per quantum to excite more than a small fraction of the chlorophyll-*a* molecules to fluorescence or to chemical reactivity. The lowered yield at 660 mμ is attributed to an analogous decline in yield of photosynthesis by chlorophyll-*b* on the long wave side of its absorption peak. An alternative explanation mentioned as a possibility by Franck (private conversation), is that part of the chlorophyll in the chloroplasts may be in a different state of combination, and with a changed absorption spectrum, but without the ability to carry on photosynthesis. Considering the wide variation of absorption spectra found in various bacteria which all give the same bacteriochlorophyll on extraction (21, 22), the possibility of chlorophyll of green plants existing in states of combination with differences in spectral absorption, as has often been claimed, should not be overlooked.

The question of the procedure for the calculation of quantum yields from S-shaped rate versus intensity curves (23) has come up again in the paper of Wassink, Katz & Dorrestein (24). These authors give quantum yield values of 0.063 to 0.12 molecules of carbon dioxide per absorbed quantum for photosynthesis in *Chromatium* strain D. They say:

If the tentative explanation of the S-shape of the curve, presented in this chapter [competition by intracellular donors which replace the hydrogen at low light intensities], should be right, then the . . . . [calculation of the yield from the maximum slope] . . . . would yield the efficiency of the process of photosynthesis with the externally supplied donor, and thus would indeed furnish the most rational answer to be obtained from the experiment.

They do not, however, state how the values reported by them were actually computed.

In an attempt to reconcile the high efficiency results in nonsulfur bacteria with the lower efficiency found in the sulfur bacteria (24, 25), Franck (private conversation) and the reviewer have recalculated the nonsulfur bacteria data [(23) and unpublished curves] on the basis of the slope of a line through the origin and tangent to the S-curve at a high intensity rather than using the maximum slope of the measured curve from which the published values were derived. From the average of the four highest efficiency experiments, obtained with pre-

treated bacteria, this different calculation gives a yield of about 0.12. The reviewer is of the opinion that this question as to the calculation of yield from sigmoid curves can be decided only by further measurements including determinations of the hydrogen to carbon dioxide ratio at various light intensities to establish without doubt the cause of the lower yield at low intensities in the actual organism used for the measurements. Direct determinations of carbon dioxide utilization rather than pressure measurements, which in this case also include hydrogen uptake, would be highly desirable.

OXYGEN EVOLUTION

Dole & Jenks (26) have corroborated by density measurements of isotope composition the previously reviewed (8) fundamental experiments showing that the oxygen of photosynthesis comes from water and not from carbon dioxide. A significant contribution to the knowledge of the step in photosynthesis which evolves oxygen was made by Fan, Stauffer & Umbreit (27) who showed that with *Chlorella* several reducible substances could be used by illuminated cells for the production of oxygen in the absence of carbon dioxide. Ferric phosphate and other ferric salts, acetaldehyde, benzaldehyde, parabanic acid, and nitrourea were found to be suitable substrates for the photochemical reaction which evolves oxygen, presumably from water. Using benzaldehyde the rate of the reaction was about half the rate of photosynthesis when carbon dioxide was supplied. Reasonable but indirect evidence is given which indicates that the reaction does not proceed through the intermediate formation of carbon dioxide from benzaldehyde. Warburg & Lüttgens (28) discovered the evolution of oxygen which takes place in illuminated sugar beet and spinach chloroplasts and also from chloroplast granules provided with quinone. This takes place according to the reaction:

$$2 \text{ quinone} + 2H_2O \xrightarrow[\text{Light}]{\text{Chloroplast substance}} 2 \text{ hydroquinone} + O_2 - 52{,}000 \text{ cals.}$$
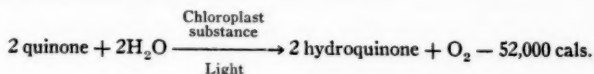
This activity was lost by dialyzing or by washing the material but could be restored nearly completely by $3.3 \times 10^{-4} M$ potassium chloride. Activation was produced by chloride, iodine, bromide, and nitrate but not by thiocyanate, sulfate, or phosphate ions. o-Phenanthroline at $10^{-4} M$ inactivated the chloroplast preparations completely indicating the participation of a heavy metal catalyst in the reaction.

According to Aronoff (private conversation) chloroplast preparations retain their ability to give oxygen evolution from an aqueous solution of quinone much better than their ability to produce oxygen from the reagents used in the Hill reaction.

The evolution of oxygen from suspensions of chloroplasts in solutions containing ferric oxalate and potassium ferricyanide was discovered by Hill & Scarisbrick (29). The possible relation of this photochemical and enzymatic reaction to the process of oxygen evolution by photosynthesis in the green leaf as well as further details of the reaction have been discussed in several papers (8, 30, 31, 32). The quantum yield of the reaction was found by French & Rabideau (33) to average about one third to one half of that for photosynthesis, but in occasional experiments it nearly approached the efficiency of true photosynthesis in *Chlorella*. The participation of an enzyme system in the Hill reaction is indicated from the thermo-lability of the preparations and also from the inhibition by low concentrations of enzyme poisons such as hydroxylamine, Duponol, fluoride, and azide. The Hill reaction is considerably less sensitive to azide than is the catalytic decomposition of hydrogen peroxide by chloroplasts (32).

Concomitant with the oxygen evolution there is also a production of hydrogen ion which affords a simple means of measuring the reaction velocity. A constant pH titration procedure has been used for the activity measurements by Holt & French (31). It was found by the titration procedure using limiting amounts of ferricyanide and running the reaction to completion that the complete utilization of 1.00 mole of ferricyanide gave 0.99 moles of hydrogen ion. Manometrically a gas production of 0.93 moles of oxygen was similarly found for every 4.00 moles of ferricyanide consumed. The ratio of hydrogen ion to oxygen produced is therefore 4 to 1. In order to obtain quantitative results it was necessary to use a red filter to prevent the effects of high light intensity on the reagents themselves. The pH optimum was found to be at pH 7.0 or 7.5 depending on the conditions of measurement. Higher rates and a greater total evolution of oxygen were found if nitrogen was used instead of air in the manometer vessels. Temperatures of about 10°C. were found most suitable for the measurements of this reaction since the activity of chloroplasts is rapidly lost at room temperature. Chloroplasts suspended in solutions of potassium ferricyanide alone were found to produce oxygen when illuminated, but at about half the rate obtained when ferric oxalate was also present. Freezing, drying, thorough dis-

integration of the chloroplasts, or precipitation by ammonium sulfate results in preparations that still retain considerable activity (32, 34). Supersonic vibration breaks up the chloroplasts to give active solutions which when centrifuged 45 minutes at *ca.* 20,000 g. are pale green and usually clear but show the Tyndall scattering effect in an intense beam of light. The ability to produce oxygen is not only a property of intact chloroplasts but also of material which has been exposed to some of the conditions that would be necessary in attempts to fractionate the substances involved in the reaction by which oxygen is produced.

Our present concept of the cellular components involved in this catalytic system is as follows: The chlorophyll presumably exists in association with a protein, "chloroplastin," and is the photosensitizer for the reaction. An enzyme which may or may not be separable from the chlorophyll-protein complex is involved and under appropriately chosen conditions is the substance whose activity limits the measured rate of the reaction. There is in addition to the chlorophyll-protein complex and the enzyme, a water-soluble factor. This water-soluble factor appears to be a different substance from the active principle in Hill's water extract of acetone-treated leaves or yeast because his extract was replaceable by ferric oxalate and was demonstrable when added to suspensions of intact chloroplasts. The new water-soluble, heat-stable factor shows its participation in addition to ferric oxalate in the Hill reaction when catalyzed by washed chloroplast fragments. Extracts giving this effect have been obtained from spinach, pea leaves, and commercial corn-steep solids (34).

Franck, Pringsheim & Lad (35) have applied the rapid and highly sensitive phosphorescence method for measurement of oxygen production (36) to a study of anaerobic photosynthesis in algae. In *Scenedesmus* the initial lag of photosynthesis following an anaerobic dark period was much shorter than that for *Chlorella* which showed several anomalies similar to those observed by fluorescence measurements (37, 38) in the rise to a constant rate of photosynthesis. The rise was much slower at 0°C. Measurements of the oxygen production by successive single flashes from photoflash lamps showed a low yield for the first two flashes in *Chlorella* but in *Scenedesmus* the first flash was as productive as the succeeding ones. In a personal communication Franck has stated, "The yield per flash, even at high concentrations of algae, is practically as great as the yield per flash measured with Emerson and Arnold's [cf. (8)] methods in air. That is im-

portant because the yield under constant illumination is so very much lower under anaerobic conditions than it is in air." Both these algae gave, at light saturation, rates of oxygen production that were much lower than those obtained when the cells had not been previously de-oxygenated. The reduction in the saturation rate was less at 0° than at 20°C. The reduction factor varies roughly from 10 with short anaerobic periods to 10,000 after long anaerobic periods. Further-more, algae that had been given a long anaerobic incubation pro-duced sigmoid curves for rate of oxygen production versus light intensity similar to those of purple bacteria. With minute amounts of algae, the yield is of the same order of magnitude as the yield in air; for instance, at low temperatures it is half as great as in air.

The same anaerobic technique was used in a study of oxygen evolution from isolated chloroplasts without added substances by Franck (39) who found an initial outburst of oxygen which dropped to a small but constant rate in two minutes. The final rate was con-stant for about an hour and was dependent on the presence of carbon dioxide but was only 0.0033 times the normal rate in intact cells. The light intensity required to give the maximum rate obtainable was 5,000 lux as compared to 12,000 lux for the intact leaves. A high intensity damaged the chloroplasts. These measurements seem to be the nearest approach to normal photosynthesis outside the living cell but the reactions of Hill and of Warburg, with added substrate, give a much higher rate and therefore seem to be more suitable for use in attempting to isolate and study the components of the oxygen evolution system. Such a procedure may, however, be used to study the natural intermediates that are replaceable for oxygen evolution, but probably not for carbohydrate formation, by quinone or by ferric oxalate.

Oxygen evolution and carbon dioxide absorption have been re-ported by Boichenko (40) in highly reducing solutions using indi-cator dyes. These experiments are of such fundamental importance that they should be repeated by quantitative methods.

FLUORESCENCE

J. Franck, French & Puck (38) and later Kautsky & U. Franck (37) have described apparatus for the rapid recording of fluores-cence intensity-time curves with which they have repeated and ex-tended some of the previous observations on the effects of varying intensity and temperature on the fluorescence curves. The alga used

by Kautsky & U. Franck, *Ulva lactuca,* was left in sea water in the apparatus for several days at a time and each measurement was preceded by a long dark-adaptation period. Their choice of material and procedure was fortunate as it resulted in a reduction of the variability of results obtained with leaves of land plants. Their data consist of families of curves of fluorescence intensity plotted against time for different exciting intensities and also for different temperatures. Separate sets of data with high, intermediate, and low recording speeds show the various "irregularities" in the curves to be systematically influenced by the external conditions.

Wassink, Katz & Dorrestein (24) have made a very thorough study of the relation between photosynthesis and fluorescence in a purple sulfur bacterium. The amount of detailed information presented is indicated by the fact that this paper contains eighty-nine figures each of which consists of a series of related curves. We will summarize here their main observations and conclusions: Data relating the concentration of hydrogen and of thiosulfate to the rate of assimilation are presented. With starved cells the saturating concentration of hydrogen was found to be at a lower pressure than in nonstarved cells. This observation appears to confirm the theory which attributes the S-shape of the intensity curves to competition at low light intensity between the internal cellular hydrogen donors and gaseous hydrogen. The general shape of the incident-intensity versus fluorescence-intensity curves under steady-state conditions is similar to those of Franck, French & Puck (38) which showed an increase in fluorescence yield with increasing incident intensity. However the choice of purple bacteria rather than of green plant material allows the introduction of another quantitatively controllable variable, the concentration of the hydrogen donor.

In the absence of added hydrogen donors the curve relating intensity of fluorescence to incident intensity is nearly a straight line with a short curved portion of much lower slope at intensities below that required for photosynthesis saturation. The addition of hydrogen, thiosulfate, or of butyrate makes the fluorescence-intensity versus incident-intensity curves much more concave upward and relatively lower at the higher intensities. The energy absorbed at the higher intensities in the presence of donors thus is seen to go into the photosynthetic process rather than being wasted as heat or fluorescence. A most important observation, that the fluorescence intensity decrease due to added donor is independent of the presence or absence of

carbon dioxide, may be interpreted as a further confirmation of the idea of the independence of the photochemical and the carbon dioxide fixation reactions. This is considered by these authors to be direct evidence for the separate nature of these processes in purple bacteria, which has already been established by Ruben *et. al.*, reviewed in (8), by Hill (41), by Fan, Stauffer & Umbreit (27), and by Warburg & Lüttgens (28) for green plants. Since the decrease in fluorescence due to hydrogen donor was independent of the amount added above a certain concentration, it was concluded that hydrogen donors such as hydrogen, thiosulfate, or butyrate were converted by an enzymatic process to some other substance which then removed the energy from the activated bacteriochlorophyll. This process was thought of as the step preceding the photochemical step. Curves are given of photosynthesis rates and of fluorescence intensity against incident intensity at a number of different temperatures. The Q_{10} for photosynthesis with thiosulfate at saturating intensity is about 3.6 at 17° to 23°C. The authors point out the necessity of determining by independent means which reaction is limiting the reaction rate in the various temperature ranges before attributing much theoretical significance to observed temperature coefficients in a process known to consist of several consecutive steps. It is, however, evident that decreasing the temperature has the same general effect on both gas exchange and fluorescence as does reducing the donor concentration. At high intensity there is a maximum rate of photosynthesis with thiosulfate at pH 6.3 of about twice the rate at pH 7.6. However, at high intensity the rate with hydrogen more than doubles in going from pH 6.3 to 7.6 but no intermediate maximum was observed. At a lower intensity the rate dropped gradually from pH 6.3 to 7.6. The effects of pH on fluorescence were consistent with the photosynthesis results. With hydrogen and thiosulfate both present in abundance, at pH 6.3 the proportion of photosynthesis attributed to hydrogen was 32 per cent of the total.

In the low-intensity region, where the S-shaped curves depicting photosynthesis rate versus intensity are changing from a flat to a steep slope, there is no abrupt change of slope of the fluorescence curve. This is taken as evidence for the utilization of intracellular organic substances as hydrogen donors at low intensity as it shows that there is no interference with the energy transfer process even though the gas exchange curve goes through a great change of slope in this region.

The influence of carbon dioxide and of added inhibitors on steady-state fluorescence at a number of intensities was investigated. A moderate concentration of carbon dioxide influenced fluorescence only when photosynthesis was permitted to take place, since no effect was obtained in the absence of a hydrogen donor, or when thiosulfate was added in the presence of cyanide sufficient to completely inhibit photosynthesis. With hydrogen donors present, carbon dioxide may either lower or raise fluorescence. These effects are explained as an influence of carbon dioxide on the step which removes the photoactivated molecules from the energy transfer system. Contrary to the direct effects of hydrogen donors on fluorescence, carbon dioxide appeared to have no such direct action on the energy transfer system in these bacteria. The effect of cyanide is analogous to that of omitting carbon dioxide in that both inhibit the dark reactions which are not directly connected with energy transfer. At high concentration cyanide reduces fluorescence by acting as an energy acceptor itself in place of hydrogen. These results all lead to the conclusion that the Blackman reaction in purple bacteria consists of at least two separate steps, each of which can be made to determine the measured rate of photosynthesis under definite conditions. Hydroxylamine acts similarly to cyanide in primarily inhibiting the dark reaction rather than influencing the energy transfer system. Strangely enough, azide acts differently than cyanide in that it inhibits at low as well as at high intensities and works directly on the photochemical system. Azide, even at low concentrations, decreases the fluorescence intensity, apparently because it has a higher affinity for the energy transfer system than do the hydrogen donors. Ethyl urethane behaves much like azide in its effects on fluorescence and photosynthesis. A figure of about 0.15 per cent at low intensity and about 0.6 per cent at high intensity is given for the fluorescence yield.

A few observations on initial changes in fluorescence are given. Here, as in the steady state, the extra variable, hydrogen donor concentration, was found to influence the fluorescence intensity. Without added donor the fluorescence increases to a constant value in three distinct stages, presumably due to the exhaustion of different intracellular hydrogen donors in the first few minutes of illumination. With added thiosulfate the fluorescence rises rapidly and declines within one minute to a constant value much as does the fluorescence of leaves and algae under normal conditions.

Dorrestein, Wassink & Katz (15) summarize the observations of

the above paper in a theoretical discussion. Briefly, their theory postulates an enzymatic system I which transforms the hydrogen donor into a form capable of combining with the bacteriochlorophyll energy transfer system. Here this substance is transformed by light energy to another substance A, which by means of another enzymatic system II reduces carbon dioxide. Temperature and pH variations are effective in changing the rate of system I. Cyanide and hydroxylamine primarily affect system II while azide and urethane attack the energy transfer system. A mathematical formulation is given. A review critically comparing the results of the Dutch group with those of Franck, French & Puck (38) is in preparation by Franck. Zscheile & Harris have measured the fluorescence spectrum of chlorophyll in various solvents and at different temperatures (42).

PLANT PIGMENTS IN RELATION TO PHOTOSYNTHESIS

The general subject of plant pigments has been well reviewed by several authors (43 to 46). The chemistry, occurrence, and function of the pigments of algae have been discussed by Cook (47); pigment separation by chromatography has been described in the book by Strain (48); and the medical uses of chlorophyll have been covered by Lesser (49). Here attention will be given only to certain aspects of such pigments as may be presumed to be directly concerned in the process of photosynthesis. It has long been believed that some carotenoids may actually use for photosynthesis the light which they absorb, but with a lower efficiency than does chlorophyll. For recent measurements supporting this view, a general discussion, and references see Emerson & Lewis (19). By measurements of the intensity of chlorophyll fluorescence of a diatom suspension illuminated with light primarily absorbed by chlorophyll as compared with the fluorescence from light of a wave length mainly absorbed by the accessory pigment fucoxanthin, Dutton, Manning & Duggar (50) established the fact that fucoxanthin acts in photosynthesis by transferring its energy to chlorophyll. Some carotenoids, possibly of photosynthetic importance, have been described by Strain, Manning & Hardin (51). Sagromsky (52) has given measurements of diatom photosynthesis at different wave lengths.

Recent papers on chlorophyll-c (chlorophyll-γ, chlorofucine) by Strain & Manning (53) and Strain, Manning & Hardin (54) describe a pigment with low absorption maxima in methanol at 618 and

580 and a high one at 450 mμ. This pigment was obtained from dino-flagellates, diatoms, and brown algae which also contain chlorophyll-*a*, but no chlorophyll-*b*. A new pigment, chlorophyll-*d*, was found by Manning & Strain (55) in red algae which do not contain either chlorophyll-*b* or -*c*. This pigment is even more interesting spectroscopically than chlorophyll-*c* because its red band is of longer wave length than that of chlorophyll-*a*. Its principal maxima are at 686, 445, and 395 mμ in ether. The maximum absorption coefficient of the red band is greater than that of the blue band. In methanol, chlorophyll-*d* has its red band at 696 as compared with 665 mμ for chlorophyll-*a*. In a methanol extract of one species of red algae about 90 per cent of the light at 710 mμ is absorbed by chlorophyll-*d* and only 10 per cent by chlorophyll-*a*. This pigment was shown to contain only magnesium oxide in its ash. Isomers of chlorophyll-*d* which were separable by chromatography were formed on standing in solution. Both chlorophyll-*c* and chlorophyll-*d* are assumed to be active in photosynthesis although such activity has not yet been demonstrated experimentally.

The participation of chlorophyll in the photoperiodic flowering response has been shown by the action spectrum measurements of Parker, Hendricks, Borthwick & Scully (56). Convenient modifications of the analytical procedures in Zscheile & Comar (57) for determination of chlorophyll-*a* and -*b* and for carotenoids have been given by Griffith & Jeffrey (58).

The work of Seybold has culminated in the publication of leaf and chloroplast absorption and reflection curves measured over the visible range with a recording spectrophotometer and in a general discussion reviewing the field (59). Using an Ulbricht sphere and a large grating monochromator Rabideau, Holt & French (60) have determined the spectral absorption and reflection curves of leaves, chloroplasts, and supersonic extracts of chloroplasts in the visible and the near infrared. The chemistry of chlorophyll has been reviewed by Stepanenko (61). Since chlorophyll takes part in the reaction which evolves oxygen it seems unlikely that it also acts by combining with carbon dioxide or with intermediates. The specific means by which chlorophyll transfers its absorbed energy remains the core of the photosynthesis problem and may now be studied in colloidal extracts of chloroplasts. The state of chlorophyll in the chloroplasts is still a fundamental but somewhat neglected problem which is discussed by Strain (62).

Miscellaneous Observations and Effects

Bacterial photosynthesis.—A review of bacterial photosynthesis showing its relation to the investigation of photosynthesis in green plants was written by van Niel (63). The same author (64) has also published a complete summary of the physiology and classification of the nonsulfur purple bacteria. Katz, Wassink & Dorrestein (65) have studied the rate of the photochemical uptake of hydrogen plus carbon dioxide at various light intensities, comparing three different concentrations of purple sulfur bacteria. As with the nonsulfur purple bacteria (23) and anaerobic algae (35), the curves relating uptake to intensity are sigmoid. Similar experiments were performed with thiosulfate as a hydrogen donor but the low intensity range was not studied in sufficient detail to decide whether or not the S-shape curve is characteristic of this type of assimilation also.

The ratio of carbon dioxide uptake to consumption of hydrogen donor (gaseous hydrogen, hydrogen sulfide, or thiosulfate) by purple sulfur bacteria has been thoroughly investigated by Wassink (66). Experiments covering a wide range of hydrogen donor and carbon dioxide concentrations showed a constant ratio of 2 molecules of hydrogen or hydrogen sulfide and 3.75 of thiosulfate to 1 of carbon dioxide by their *Chromatium* strain D. Unfortunately all these measurements were made at high light intensities thus leaving the theory of internal donor competition without the experimental support which such determinations could have given if made also at lower intensities. The measurements were made by adding small amounts of the donor to the suspensions in the presence of an excess of carbon dioxide or vice versa. Measurements were made of the initial reaction rate as well as of the total gas uptake. This allowed the relation of hydrogen donor concentration to the rate of photosynthesis to be determined in addition to the stoichiometric relations. This information is obtainable only with bacterial or certain algal photosynthetic systems and not with higher plants which use water as a hydrogen donor.

The effect of light on Chlorella respiration.—Emerson & Lewis (19) discovered a stimulation of oxygen consumption in the dark by a previous exposure of the *Chlorella* to light. This was caused only by blue-green light. There was no such effect with light of longer wave length than 530 or shorter than 435 mμ. The maximum stimulation was in the range 470 to 480 mμ. The wave length specificity

points to a carotinoid as the light-absorbing pigment whose activation is responsible for this increased rate of oxygen consumption. This stimulation of respiration by short exposures of *Chlorella* to blue-green light was carefully distinguished from the increase of respiration due to the accumulation of photosynthetic products which is caused, particularly in starved cells, by any light capable of causing photosynthesis. Other work on the effects of radiation on plant respiration has been reviewed by Weintraub (67).

The products of photosynthesis.—A molecular equivalence between the carbon dioxide uptake and the carbohydrate which was formed has been found by Smith (68) with sunflower leaves which is in accordance with the usual stoichiometric equation for photosynthesis. Starch and sucrose appear to be formed simultaneously from a common precursor. At 10°C. more sucrose and less starch and monosaccharide were formed than at 20°C.

Nitrate assimilation was found by Burström (69, 70) to be dependent on photosynthesis and not to be correlated with respiration since it takes place only in leaves that are illuminated. In the light, nitrate reacts with some intermediate product of photosynthesis to form a compound containing nitrogen and carbon.

Inhibitors.—Gaffron (71) has found that derivatives of vitamin K which stimulate respiration will inhibit photosynthesis and will prevent anaerobically adapted algae from producing oxygen in bright light. These poisons also stopped the adaptation which is necessary for photoreduction of carbon dioxide by hydrogen but they did not poison that process itself. Hydroxylamine, *o*-phenanthroline, and vitamin-K derivatives cut the rate of photoreduction to exactly half its normal value but no further even with higher concentrations.

Phosphate esters in photosynthesis.—An hypothesis, based on known enzyme reactions, involving a cycle of phosphorylation of a carboxyl group, reductive carboxylation, and hydrogenation to a hydroxy acid has been proposed by Lipmann & Tuttle (72). Comments on other phosphorylation theories in photosynthesis and phosphate esters in plants can be found in several recent papers (11, 12, 73 to 76).

EXPERIMENTAL TECHNIQUES

The cultivation of algae.—An automatic dilution device for culturing unicellular algae in a medium of constant composition and with a constant culture density throughout growth has been described by

Myers & Clark (77). Over a period of twenty-one days the samples removed daily gave an absolute photosynthetic rate whose greatest deviation from the mean was 4 per cent. The apparatus may be used not only for providing a continuous supply of algae for photosynthetic experiments, but is also designed for studies of the effects of culture conditions on the growth rate of algae. Various papers (78 to 82) on the cultivation of *Chlorella* in different solutions and the effects of different growth media on physiological processes are of considerable value to those concerned with the cultivation of *Chlorella* for photosynthesis investigations.

The measurement of gas exchange.—A new method with a very small time lag for measuring traces of oxygen under anaerobic conditions (about 10^{-3} mm. oxygen pressure) based on the quenching by oxygen of phosphorescence of a dye adsorbed on silica has been described by Pollak, Pringsheim & Terwoord (36). This method measures with an accuracy of a few per cent 1.5×10^{-4} c.mm. of oxygen produced from a single flash of light. Its accurate range is restricted, with the particular dye chosen, to extremely low oxygen pressures. Voltametric measurements of oxygen in solution are discussed by Müller (83), Kolthoff & Laitinen (84), and Manning (85).

Infrared absorption measurements of carbon dioxide have been made by Veingerov (86) and an infrared gas analysis machine is commercially available (87). Automatic recording of carbon dioxide by conductivity is described by Leach, Moir & Batho (88); Warburg's manometric procedures have been described with many helpful technical details in a book by Umbreit, Burris & Stauffer (89). It is to be hoped that electrical pressure gauges can be adapted to studies of tissue metabolism in such a way as to provide for automatic recording of pressures in a series of vessels. The use of long-lived radioactive C^{14} in photosynthesis may soon be possible.

CONCLUDING REMARKS

Until the last few years photosynthesis has been considered as a relatively simple system of a few closely co-ordinated reactions which might be analyzed kinetically by rate measurements under different conditions of carbon dioxide pressure, temperature, light intensity, etc., in the same way that the molecular mechanisms of many photochemical reactions have been established. It now seems that this approach, if only applied to the over-all reaction, may be inadequate to lead to a complete description of the process of photosynthesis. The

tendency at present is to attempt to study one major step at a time in the hope that kinetic measurements in connection with the use of artificial substrates, isolation procedures, and chemical analyses may uncover the molecular mechanisms of the single separable partial reactions, one at a time. These major steps now most easily separable experimentally are the following: (*a*) carbon dioxide fixation which is open to investigation by tracers; (*b*) photochemical oxygen evolution by chloroplast material from artificial substrates which presumably is linked normally with the formation of a reducing substance which later acts on some product of reaction (*a*); (*c*) changes in the distribution of the different phosphate compounds in illuminated cells which may provide a mechanism for linking steps (*a*) and (*b*); and (*d*) changes in the energy acceptors in the neighborhood of excited chlorophyll molecules which are measurable by their quenching effects on fluorescence. In brief one might say that the field is ripe for attack by biochemical methods since reactions have been discovered that take place in disintegrated material.

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THE RESPIRATION OF PLANTS

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As the respiration of plants has not previously been reviewed in these pages, it seems desirable to look further back than the last twelve months. The subject is a wide one, however, so only the more purely biochemical aspects of its mechanism can be dealt with, and those but briefly.

Respiration rates of plant tissues depend on both their carbohydrate and nitrogen contents (1). It is fairly generally held that active and healthy plant tissues normally consume carbohydrates in respiration and in doing so build their nitrogen up to proteins, which in their turn provide the catalytic machinery for further activity (2, 3). It is further commonly believed that the sugars consumed in respiration undergo a cleavage that is followed by oxidation of the cleavage products. Attempts that have been made from time to time to show that the sugars are themselves oxidised in plant respiration have not convinced the majority of investigators. A fairly recent attempt by Boysen-Jensen (4) depended upon the ability of monoiodoacetic acid to differentiate between anaerobic cleavage and aerobic oxidation by specific poisoning of glycolysis. It has been noted that iodoacetate, unlike many other specific inhibitors, takes a relatively long time to develop its full effect (5). Turner (6) has shown that the time taken is longer in air than in nitrogen, and that the apparent failure of iodoacetate to poison aerobic respiration of carrot slices is only temporary, a high degree of inhibition developing after seven to eight hours. Even in *Aspergillus niger,* the classic fungal source of Müller's glucose oxidase, it appears that glucose oxidation may be exceptional rather than the rule (7).

Accepting these outlines of the subject, four main topics should be considered, (*a*) glycolysis, by which is implied the cleavage of sugars to whatever product, (*b*) oxidation mechanism, (*c*) the relations between oxidation and glycolysis, and (*d*) the role of nitrogen. The first three are dealt with here; the fourth was reviewed last year (3).

GLYCOLYSIS

Sugar complex.—The three sugars, glucose, fructose, and sucrose, are ubiquitous in healthy plant tissues. As respirable materials they are frequently supplemented by starch, and less frequently by hemicelluloses, fructose anhydrides (8), raffinose (9, 10), organic acids (11), etc. Fats are characteristic of reproductive bodies such as seeds and spores (12). Under the influence of the cell catalysts the sugars and their anhydrides appear to be interchangeable with ease. Some of the mechanisms involved have been recently reviewed, e.g., by Hanes (13) and Hassid (14) for starch, and by Archbold (8) for fructosans. The only question that can be considered here is the form in which material is drawn from the complex for respiration. The old categories of starch and sugar leaves emphasize the predominance of starch and sucrose as materials for respiration. It has long been known that, while the products of starch degradation *in vitro,* whether by enzymes or acids, are maltose and glucose, the principal product in the living system is sucrose. Thus, although sucrose respiration may occur in leaves and other organs that form no starch, starch respiration does not occur in the absence of sucrose. The relation of sucrose to starch, so long a puzzle, and of both to respiration, may have found an explanation in the action of the phosphorylases catalysing the reversible reactions starch $\rightleftharpoons$ glucose-1-phosphate (15) and glucose-1-phosphate $\rightleftharpoons$ sucrose (16). Since glucose-1-phosphate can lead to other esters in a respiratory sequence, starch and sucrose appear as complementary carbon reservoirs. Respiration has in some respects a more direct connection with starch and sucrose than with the hexoses. Under certain conditions respiration rate becomes a function of sucrose concentration (17, 18, 19). Onslow (20) examining the literature available in 1931 came to the conclusion that, when sucrose is available and starch absent, the amount of sugar respired is approximately equal to half the sucrose disappearing. Direct experiment (21) with Bramley's Seedling apples convinced her that in the starch-free phase there was approximate equality between the hexose equivalents of total sugar loss, carbon dioxide emission, and sucrose-loss/2. Onslow assumed that the moiety of the sucrose disappearing would be the relatively active fructofuranose and the half accumulating the more inert glucopyranose. In general, however, it is fructopyranose that accumulates in maturing fruits and Archbold & Widdowson (22) showed this to be true for Bramley's Seedling apples

in particular. Nevertheless, it is observable that in phases of mild starvation furanose anhydrides—sucrose, raffinose, fructosans, etc.—are consumed, while pyranoses—glucose, fructose, and maltose—temporarily accumulate. This is noticeable in the data of Yemm for barley leaves (19), of James for barley embryos (10), and of Barnell for banana pulp (23). It appears to be true also of Belval's results for fructosan in rye stalks (24), though the situation is complicated by translocation into the ripening ear.

The special respiratory position of starch and the furanosides, particularly sucrose, is not difficult to explain. Their phosphorylation is reversible (15, 16) and therefore occurs with little change of free energy, and no linkage with an exergonic reaction is essential. This is in marked contrast with the requirements of the hexoses.

If we assume with Lipmann (25) that the energy of the phosphate-glucose linkage must be similar to the glucose-glucose linkage in glycogen (or starch) with which it is in equilibrium, it will be approximately 3 kcal. At least this amount of energy must therefore be supplied in forming glucose-1-phosphate from a free pyranose. Actually more energy will be needed if, as is shown later to be probable, the mechanism utilises adenosine esters as carriers of the phosphate, since an energy peak of 11 kcals must be passed in the synthesis of adenosinetriphosphate. This must be provided by a suitable linkage with some "energy producing" phase of the respiratory mechanism [cf. (26)]. Nothing is yet known about the phosphorylation of maltose; but its much greater resistance to hydrolysis suggests that it may also be less readily phosphorylised than the furanosides. The special contribution of the furanose structure in facilitating respiration appears to lie in the provision of more readily phosphorylated groupings, rather than in providing a structure which is in itself more easily decomposed.

Phosphorylation cycle.—The phosphorylases are accompanied in the living cell by hydrolases (amylase, invertase, phosphatase, etc.) which act on the same substrates and, at least in extracts, are in competition with the phosphorylases (27, 28). The question arises whether phosphorylation is indeed an essential feature of the plant's respiratory mechanism. From the details enumerated below it is now clear that plant preparations are able to perform a cycle resembling that familiar in yeast. James & Arney (29) concluded it to be a normal part of respiration in barley from the following facts: (*a*) The carbon dioxide emission of phosphate-starved embryos showed a linear

relation with phosphate supply; (b) normal seedlings showed a positive correlation between respiration rate and esterified phosphate; and (c) during starvation the phosphate ester content and the respiration rate declined simultaneously. A similar parallel is reported (30) between respiration rate and phosphate supply in the leaves of phosphate-starved barley plants. In both embryos and leaves the effect occurred only in the presence of an adequate sugar supply. Addition of hexosephosphates to the medium also increased the oxygen consumption of *Chlorella* (31).

The phosphate-sugar esters to be expected if a cycle is in operation have been identified in plant tissues and consist chiefly of mixed hexose-6-phosphates (28, 32, 33, 34). Hexose-1,6-diphosphate accumulates only in small quantities if at all (28, 35). Small quantities of "7-minute phosphorus" suggesting the presence of a labile carrier have also been found (28, 36, 37), as well as a highly resistant ester, possibly phosphoglycerate (37).

Esterification of inorganic phosphate has been shown to occur in pea meal (38) and cell-free extracts (39) with formation of hexose-diphosphates and hexose-monophosphates. The esterification is probably catalysed by adenosine esters as it has been found that the eventual formation of pyruvate (40) and carbon dioxide (41) is increased in such systems by addition of adenylic acid. Glucose as well as sucrose is esterified.

The decomposition of hexose-1,6-diphosphate in preparations from tobacco leaves (42) and barley seedlings (43) has been established. A zymohexase, with a general resemblance to muscle zymohexase, has been obtained from potatoes and from pea meal (27, 44). Mixed triosephosphates have been identified as products of the breakdown, especially in the presence of cyanide and bisulphite (27, 43, 44). Addition of $0.025\,M$ sodium fluoride to barley saps with added hexose-diphosphate leads to the accumulation of an ester highly resistant to hydrolysis and with solubility properties suggesting phosphoglycerate (43). Both hexosediphosphate and phosphoglycerate are converted by cell-free barley saps to pyruvate, which accumulates if 1-naphthol-2-sulphonic acid is added to reduce carboxylase activity. This formation of pyruvate is prevented by fluoride (40). Conversion of phosphoglycerate to pyruvate has been observed in bean and pea preparations (45). Such data seem to make it evident that the higher plants possess a phosphorylating mechanism capable of glycolysing sugars and polysaccharides to pyruvate. The mechanism must be similar, at least in

outline, to the mechanisms present in muscle and yeast. Pyruvate itself has been isolated as the 2,4-dinitrophenylhydrazone from living barley leaves poisoned with 0.2 per cent 1-naphthol-2-sulphonic acid (46). It is noteworthy that all these reactions occur aerobically and in the author's laboratory it has been a common practice to examine them without exclusion of oxygen.

Carboxylase activity.—Feeding barley leaves and embryos with pyruvic acid leads to an increased rate of carbon dioxide production with a rise of the R.Q. (respiratory quotient) towards 1.2 (47). It has long been held that an active carboxylase, similar to that of yeast, occurs in the higher plants. In some of the earlier experiments, where very long incubation periods were used, precautions to exclude yeast and bacterial contaminations may not always have been adequate; but the more recent investigations appear to have supplied satisfactory identification of the enzyme in pea roots (48), oats (49), barley seedlings (47, 50), and lily pollen (51). The enzyme is either preformed in the dormant barley grain or arises to full activity immediately upon soaking (47), contrasting, in this respect, with its absence from dormant *Neurospora* spores, and its development during their germination (52). A separable cocarboxylase is reported from a variety of plant tissues (49, 50, 53). James & Norval (47) were able to confirm the older results showing acetaldehyde and carbon dioxide to be co-products of the reaction, and observed the formation of acetaldehyde even in the presence of atmospheric oxygen. The presence of acetaldehyde in plant tissues, even under aerobic conditions, has frequently been reported, especially in fleshy fruits (54, 55, 56). Niethammer (57) identified acetaldehyde by means of the nitrophenylhydrazone in actively germinating and respiring seedlings of maize, *Oenothera,* and *Secale* and showed that it was absent from dormant and nonviable seeds and from seeds undergoing dormant maturation.

Alcohol formation.—Under anaerobic conditions or in the presence of cyanide (55) alcohol may appear in plant tissues; presumably by reduction of acetaldehyde. No data seem to exist for the value of the ratio, sugar consumed/alcohol formed. A search through the older literature soon shows that a wide range of values exists for the ratio alcohol formed/carbon dioxide emitted. Although among more recent results (58, 59) carrot discs are reputed to form a molecule of alcohol for each molecule of carbon dioxide released, Fidler (60) in an extensive series of determinations upon apples obtained ratios varying from 0.97 to 0.47. Wetzel (58) found that the ratio in carrots, although

it started near 1.0, soon fell away and had reached 0.51 after 64 hours. It would seem, therefore, that calculations based on the application of the alcoholic fermentation equation to the higher plants and seeking to give numerical values for Pasteur effects, Meyerhof quotients, and the like from measurements of carbon dioxide emission alone are based at present on a somewhat precarious footing. Thomas & Fidler (61) cautiously suggest that approximate values may sometimes be determined if alcohol estimations are undertaken in addition. The causes of the discrepancy are still uncertain; but it may be pointed out that even where an alcohol/carbon dioxide ratio of 1 is obtained it is still far from being evidence that anaerobic respiration consists solely of an alcohol fermentation. It has been shown (62) that lactic acid occurs both aerobically and anaerobically in a wide variety of plant tissues. Its anaerobic accumulation would cause a consumption of sugar without affecting either side of the alcohol/carbon dioxide ratio.

OXIDATION MECHANISMS

Dehydrogenases.—Living plant tissues under nitrogen decolorise methylene blue, which is reoxidised inside the tissues when they are returned to air (63, 64, 65). The respiration of the tissues is greatly accelerated and the acceleration is not abolished by washing (31). The respiration of tobacco stems (5) is strongly inhibited by malachite green, a reputed dehydrogenase poison. The dehydrogenases responsible for these effects are not always readily extracted and often appear reluctant, when extracted, to react with methylene blue. For these reasons, perhaps, the establishment of their nature and wide occurrence was rather long delayed. By the use of thionine, indigotetrasulphonate, 2,6-dichlorophenolindophenol, and other redox dyes the occurrence of the following dehydrogenases has now been shown in a variety of plants and their parts: glucose (51), citric (51, 66 to 69), isocitric (66, 70, 71), malic (51, 67), succinic (51, 72, 73), glutamic (51, 70), glycerophosphoric (67), oxalic (74), lactic (51, 63), formic (75), and alcohol (67, 75). At least the alcohol (67, 75), formic (75), and malic (67, 70) dehydrogenases are known to be able to co-operate with coenzyme I as extracted from yeast. Alcohol dehydrogenase cannot be a very effective oxidising enzyme in most plants since alcohol, once formed, is usually removed very slowly if at all. The enzyme responsible for alcohol formation, which may or may not be alcohol dehydrogenase, appears to be more sensitive to cyanide poison-

ing than is usual for a dehydrogenase (55). The lactic dehydrogenase of barley (63) resembles that of yeast in being active in extracts without the addition of coenzyme; but the coenzyme appears to participate in the oxidation of triosephosphate by preparations from the same species (43).

An oxalic "aerobic dehydrogenase," i.e., an enzyme reducing oxygen and not reducing other hydrogen acceptors, and not poisoned by cyanide, azide, or sulphide, is reported by Franke & Hasse (76) from the moss *Hylocomium umbratum*. It requires no coenzyme and no pigmented carrier and is unusually resistant to destruction. The authors state that the main difficulty in its investigation lies in its extreme activity. The nearest analogues appear to be the fungal enzymes, glucose oxidase, and notatin (77). The formic dehydrogenase of pea-meal (75) and alcohol dehydrogenase of pollen (51) are able to co-operate with flavoprotein. Riboflavin is reported from many plant sources (78 to 81). The relation of these catalysts, however, to plant respiration is still quite obscure. It is observable that a fraction of the respiratory oxygen uptake is usually resistant to poisoning with dilute cyanide (31, 82 to 86), and that in some adult tissues resistance becomes very high (85, 86). It would be rash to conclude at present that such respirations are necessarily operated by flavoprotein systems. Results obtained in "specific poisoning" experiments need to be interpreted with great caution unless supported by other lines of evidence and, particularly until the poison has been tested upon the enzyme as extracted from the tissue concerned. Great variation exists, for example, in the resistance to cyanide poisoning between the catechol oxidases from potatoes and from apples (87). Iodoacetate poisons the alcohol dehydrogenase of yeast (88) and of oat coleoptiles (89), but not that of animal tissues (88).

Catechol oxidase.—The occurrence of three cyanide sensitive oxidases reducing atmospheric oxygen, together with their appropriate carriers, is now well established for the higher plants. In order of their discovery they are the catechol (polyphenol), cytochrome, and ascorbic acid systems. The mechanism of the catechol system has recently been reviewed (90, 91) and only its relation to plant respiration needs to be considered here. The old argument that catechol oxidase could not be a respiratory enzyme because its occurrence is not universal appears to the present writer to have no weight. In spite of claims that have been made on behalf of the cytochrome system, the effect of the data now available is strongly in favour of the supposition that

different plant tissues achieve the same ends by slightly different means and that the means may be changed even during the existence of a single tissue.

The view has been advanced (92, 93) that catechol oxidase, together with its associated orthodihydroxy compound, which has been extracted but not purified, is responsible for the greater part of the respiratory oxygen uptake of potatoes and similar tissues. Although catechol itself, after a brief stimulation inhibits the respiration of tissue slices, the catechol compounds, protocatechuic acid, caffeic acid, dihydroxyphenylalanine (dopa), and gallic acid, are oxidised without complications. They accelerate the respiration of potato "permanently" with a normal R.Q. of 1. Protocatechuic acid (93) is oxidised much more slowly than catechol and causes no discolouration of the tissues, so its quinone is presumably again reduced. It appears, in short, that all these substituted catechols are capable of acting as redox bodies in association with catechol oxidase in the respiratory system of potatoes.

The relations of the catechol system with amino acids is noteworthy. Using potato slices Boswell (94) found that caffeic acid co-operated in the oxidation of glycine, glutamic, and aspartic acids, but did not co-operate in the breakdown of nonamino acids such as malic. Similar action by the extracted catechol system was already familiar (95). It is not necessary to conclude that this is the normal respiratory activity of the system. A connection with normal carbohydrate respiration is suggested by its ability to oxidise ascorbic acid (96, 97) and reduced coenzyme I (98). As will be shown later these are much more probable links between the catechol system and respiratory substrates than such a substance as malic acid. Further, it is not evident why an R.Q. of 1 should be observed if the only action were an oxidative deamination. However, we may well have here an explanation of the protective action of carbohydrates upon tissue proteins. So long as carbohydrate respiration is functioning normally, the carriers are maintained in the reduced state (99, 100) and hence also the natural substituted catechols. With the exhaustion of the substrates the intermediate hydrogen carriers swing over to their oxidised forms and the o-quinones begin to oxidise amino acids. An analogous system has been realised by Robinson & Nelson (101) with potato extracts containing tyrosine and the enzyme tyrosinase, which Nelson and his collaborators regard as identical with catechol oxidase. Tyrosine may be irreversibly oxidised to the corresponding 3,4-dihydroxyphenylalanine (dopa). Further oxidation to the o-quinone occurs and, in the

extracted system, may lead to melanin formation. With ascorbic acid the dopa is maintained in the reduced form and tyrosine protected from further oxidation. In some such way as this catechol oxidase (tyrosinase, polyphenol oxidase) may well be effective in plant respiration.

Cytochrome oxidase.—After Keilin's original discovery of cytochrome in a number of plant tissues, Bhagvat (102, 103) extended the examination to a considerably wider range. She found the four characteristic absorption bands of cytochromes *a, b,* and *c* in all the tissues examined, viz., in embryos of cereals and legumes, roots of dandelion, seakale, turnips and parsnips, leaf bases of celery and leeks, onion bulbs, and flower buds of rhubarb. Shaking with air caused the bands to disappear, succinate caused reappearance, and reoxidation was prevented by the presence of cyanide and azide. Cytochrome-*c* from ox heart was rapidly reduced by ground-up plant tissues poisoned with cyanide. The writer (unpublished) has been able to confirm these observations with barley embryos, one of the materials used by Bhagvat, and also the occurrence of cytochrome oxidase as determined manometrically. The Q_{O_2} values were comparable with those obtained for animal tissues. Most of the tissues examined by Bhagvat showed no catechol oxidase activity; it was present, however, as well as the cytochrome system, in parsnips, celery, turnips, and sugar beet. The occurrence of the cytochrome system has also been reported in pollen (51), oranges (104), soybeans, green leaves, cruciferous inflorescences and roots, seaweeds (105), and *Aspergillus* (106). Owing to the very low concentrations of cytochrome in plant tissues, about one twentieth of its concentration in yeast (102), its spectroscopic identification is difficult and not always certain. A crude preparation of cytochrome-*c* was obtained by Yakashuji (105) from the seaweed *Pórphyra tenera;* but the outstanding contribution in this field has been Goddard's isolation of a purified cytochrome-*c* from commercial wheat germ by a new method (73), yielding 4.6 mg. per kilogram of germ. The purified preparation was spectroscopically identical with ox heart cytochrome-*c,* was oxidised, in low concentrations, at the same rate by pig heart cytochrome oxidase, and was reduced by reductase prepared by Haas' method. An active cytochrome oxidase was prepared also from wheat germ.

Nevertheless it seems certain that the cytochrome system cannot play a universal role in plant respiration. Sreerangachar (107), after a careful examination, came to the conclusion that cytochrome is not

present in the leaves and stalks of the tea plant. No absorption bands could be detected in material decolorised with acetone, and no cytochrome could be extracted by the method of Keilin & Hartree. A highly active oxidase concentrate would not quench the absorption bands of reduced goat heart cytochrome in twenty-four hours and there was no uptake of oxygen manometrically. The enzyme proved to be a catechol oxidase with an activity proportional to its copper content. Iron was absent from the purified enzyme. Although cytochrome activity occurs in the submerged hyphae of *Aspergillus niger* cultures, there is little or none in surface hyphae (106). Goddard and his collaborators have found that, although the respiration of barley seedlings and carrot roots (84, 85) is poisoned by cyanide, azide, and carbon monoxide, the respiration of mature leaves of these species and of wheat (108) is not poisoned. It can hardly, therefore, depend upon a cytochrome oxidase. It is possible that all plant tissues possess a slow "basal respiration" unaffected by cyanide (cf. p. 423). It has been claimed that, even in yeast, continued culture in the presence of cyanide develops a strain that possesses no cytochrome oxidase (109). It cannot be assumed, as it sometimes is, that any respiration of a plant tissue inhibited by cyanide is necessarily due to the cytochrome system. Catechol oxidase is also poisoned by cyanide, sulphide, and carbon monoxide, though the last effect is not reversible by light (110, 111). The ascorbic oxidase of barley is not inhibited by azide or carbon monoxide (writer, unpublished), but is 100 per cent inhibited by $10^{-3} M$ cyanide (63, 112). Some enzymes which contain no heavy metal at all are also poisoned by cyanide (113, 114) possibly due to cyanhydrin formation. It is at least necessary to demonstrate a light-reversible inhibition by carbon monoxide before participation of cytochrome oxidase is indicated.

Ascorbic oxidase.—Combined researches from many angles show that ascorbic acid (vitamin C) is universally distributed in plant tissues (99, 115, 116, 117). It is known to increase during the active stages of germination of oats (115), rice (118), barley (119), tomatoes (120), peas (115, 120), and during the sprouting of potatoes (122, 123). It is normally in the reduced form and is rapidly oxidised when the tissues are damaged (99, 120). Oxidation may be due to the catechol system (96, 97), the cytochrome system (124, 125), peroxidase in the presence of orthodihydroxy compounds (126, 127), or to a specific ascorbic oxidase (63, 112, 128 to 131). The last appears to be a copper-protein complex (132 to 136) though inorganic copper is

also well known to catalyse the oxidation. The suggestion that biological oxidation of ascorbic acid is due to inorganic copper (137) does not seem as yet to be satisfactorily established, since the biological oxidation shows partial specificity for *l*-isomers (138) and amino acids, proteins, and sodium chloride inhibit the inorganic but not the enzymic copper catalysis (139, 140, 141). Inert protein is, indeed, necessary to maintain the activity of the enzyme (134). The ascorbic oxidase of cauliflower, cabbage, *Cucurbita pepo* (139), and barley (James & Harper, unpublished) is strongly inhibited by the copper precipitants diethyldithiocarbamate, potassium ethyl xanthate, and 8-hydroxyquinoline. Diethyldithiocarbamate is also known to inhibit catechol oxidase (110) but it inhibits cytochrome oxidase only very slightly.

Oxidation of ascorbic to dehydroascorbic acid is reversible and Szent-Gyorgyi first suggested that it might behave as a redox body in plant respiration. The hypothesis was unsubstantiated for some time because no suitable mechanism for the reduction was known in plant tissues. James & Cragg (63) have now shown that barley saps catalyse the oxidation of lactic to pyruvic acid by means of the ascorbic system. The presence of lactate maintained the ascorbic acid in reduced form, and a continuous oxidation system was set up at the expense of the lactate. Pyruvate was isolated from the mixture at the end of the reaction. Other α-hydroxyacids, glycollic and tartaric, were oxidised, but more slowly. Sucrose, alcohol, and acetaldehyde were not oxidised and malate not always. It is not yet known whether addition of coenzyme I would bring about oxidation of the latter substances (cf. p. 422). The reduction of dehydroascorbic acid by glutathione is catalysed by many plant extracts (112, 142), but the relation of glutathione to plant respiration, if any, is still as obscure as ever. It is not reduced by lactate in barley saps (143).

Dicarboxylic and tricarboxylic acid cycles.—Malic and citric acids are of common and perhaps universal occurrence in plant tissues. Succinic, fumaric (144, 145), and isocitric acids (146, 147) have also been found, but *cis*-aconitic, which occurs in the modified Krebs cycle, has been sought in vain (147). Malic, succinic, and isocitric dehydrogenases also occur, as well as fumarase and aconitase (67) and it seems clear that the acids are readily interconvertible. A vast amount of labour, reviewed by Bennet-Clark (11), has been expended upon their complex behaviour in plant tissues. Up to the present no evidence has accrued that they act catalytically in hydrogen transfer, and Ben-

net-Clark & Bexon (148) have recently found strong evidence to the contrary in the respiration of beetroot discs. Malate, succinate, and citrate all caused increased respiration rates, but the increase was always accompanied by an abnormal rise of the R.Q. to 1.5 to 2.3. The extra respiration was not inhibited by malonate and, so far from the system acting catalytically, the extra carbon dioxide formation and oxygen consumption were equivalent only to a small fraction of the acid consumed. Participation of the acids in a Krebs or Szent-Gyorgyi cycle seems clearly to be excluded. The normal respiration of tomato roots is also known to be resistant to malonate poisoning (149) and unpublished work with carrot discs is said to have yielded a similar result (86). Machlis (150) found that malonate inhibited both oxygen and carbon dioxide exchanges in the respiration of barley roots but the oxygen depression was not reversed by succinate, fumarate, or citrate and only to a small extent by malate. Carbon dioxide emission in the presence of malonate was only slightly increased by the same acids. The significance of the malonate inhibition is therefore at present uncertain. Malate and citrate caused no increase of oxygen uptake by *Chlorella* (31).

Relations between Oxidation and Glycolysis

Glycolysis provides a long series of potential substrates for oxidation. In certain conditions, especially in aging fruits, the whole series down to alcohol may survive in air, at least to some extent (54, 56). Various intermediate products of glycolysis have been shown to be formed in more normal tissues, as detailed in the first section. The most probable picture at the present time represents respiration as including the interruption of glycolysis by oxidation at several possible stages, perhaps by more than one mechanism of hydrogen transfer. As mentioned earlier, it seems improbable that any considerable oxidation of the sugars themselves takes place; and attempts have been made to localise the point or points at which oxidation interrupts the glycolytic train by the application of inhibitors, especially iodoacetate, to actively respiring tissues. Unfortunately, the action of iodoacetate is not very clearly defined, though probably it is most considerable upon the oxidation of triosephosphate. Triosephosphate accumulates in iodoacetate-poisoned barley digests containing hexosediphosphate. Iodoacetate also inhibits the alcohol dehydrogenase of oat coleoptiles and yeast (p. 423), though not so strongly. Inhibition of glyoxalase and the partial inhibition of phosphorylation, reported for

other organisms, do not seem to have been tested with enzymes from the higher plants. It seems probable that the sensitivity of plant respiration to iodoacetate poisoning (p. 417) implies that aerobic glycolysis normally proceeds at least as far as the triosephosphate stage. This is confirmed by the fact that oxygen uptake is affected (149) as well as carbon dioxide output.

Under anaerobic conditions it may be supposed that triosephosphate is oxidised at the expense of acetaldehyde or pyruvic acid, since both the latter—and their reduction products, alcohol and lactic acid— are known to be formed. In air, the hydrogen is transferred to oxygen instead, and the production of lactate and alcohol suppressed more or less completely. Evidence has been brought forward (43) which indicates that, in barley preparations, the oxidation of the triosephosphate is catalysed by the ascorbic system. Ascorbic acid was found to accelerate the conversion of hexosediphosphate to phosphoglycerate, and the effect was abolished by traces of cupric ion, due apparently, to irreversible oxidation of the ascorbic acid. When the oxidation system was poisoned with iodoacetate, fluoride, or cupric ion, alkali-labile phosphate (triosephosphate) accumulated in the digests. Cyanide and bisulphite as fixitives caused even larger accumulations. At the same time it was shown that hexosediphosphate caused an oxygen consumption only if ascorbic acid was present, and then large increases occurred. The series of reactions was further accelerated by coenzyme I. It seems, therefore, that oxidation of triosephosphate was occurring by transfer of hydrogen through the system coenzyme I, dehydrogenase, ascorbic acid, ascorbic oxidase, oxygen. An analogous oxidation of triosephosphate from hexosediphosphate, by means of the cytochrome system, has been carried out with preparations from pollen (51). In these two examples a reasonably complete connection has been established between a major glycolytic product and a well-defined oxidation system. Lactic acid is also oxidised by the ascorbic system of barley saps, but the respiratory status of lactate is still uncertain (63). There is some slight evidence that it may be oxidised in the respiration of seedlings (62, 151, 152); and the ascorbic system would be available for the purpose. Oxidation of pyruvic acid is not yet known to occur in higher plants, and acetaldehyde, derived from the decarboxylation of pyruvic acid, affords the most important possibility for further oxidation. Alcohol is not readily oxidised in plants (31, 55) and, in any case, would presumably be reoxidised via acetaldehyde. Very dilute applications of acetaldehyde vapour have been observed to

increase the carbon dioxide output of apples and oranges (153, 154) and at the same time to decrease their net sugar consumption. At higher concentrations, the acetaldehyde begins to compete with oxygen as hydrogen acceptor, and alcohol appears in the tissues. Nothing is known as to the system by which acetaldehyde is oxidised, and very little about further stages and products of oxidation. Oxidation of alcohol has been catalysed *in vitro* by the catechol system plus co-enzyme I (98). Since a Pasteur effect is considered to occur in many plant tissues (83, 155, 156) it is possible that some at least of the acetaldehyde may be involved in resynthesis. The sparing of sugar, noted above as resulting from feeding with acetaldehyde, might be attributable to such a cause. There are, however, other possible paths of resynthesis and other possible explanations of the Pasteur effect (157, 158), and it must be admitted that no progress has been made in this connection comparable with the progress of the last decade in other branches of the subject.

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THE BIOCHEMISTRY OF YEAST

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Mineral Requirements

Various aspects of the biochemistry of yeast are presented in a review by Joslyn (1). The composition of the ash, the mineral requirements for yeast growth, the toxic and antagonistic effects of different salts, and the metabolism of the more common inorganic and trace elements are described. Although an attempt is made to show the dispensable or indispensable nature of the principal elements, it is evident that our knowledge of the mineral metabolism of yeast is very limited.

Further information as to the indispensable nature of copper, cobalt, manganese, and zinc as well as the function of the various metallo-proteins and enzyme complexes is presented by Green (2). The exchange of magnesium with calcium, manganese, cobalt, and nickel in yeast carboxylase, normally a diphosphothiamine-magnesium-protein compound, is also discussed.

Booy (3) found that thallium, beryllium, zinc, cadmium, lead, manganese, iron, nickel, cobalt, aluminum, tellurium, lanthanum, and cerium reduced fermentation at concentrations below $1 N$. At greater concentrations, however, they become inhibiting. Lithium, sodium, potassium, rubidium, cesium, ammonium, magnesium, calcium, barium and strontium either stimulate or have no effect on fermentation up to about $1 N$. Mercury, silver, copper and uranous oxide salts suppress the degradation of sugar in low concentrations.

It was early observed (4) that the potassium ion is indispensable for some partial processes of fermentation. This fact has been confirmed time and again by later investigations, which now give an account of its particular role. In the first phase, according to Pulver & Verzar (5) 1 gm. yeast with 10 mg. glucose takes up 0.5 mg. potassium. As soon as the carbon dioxide production starts, potassium is set free. In experiments with small amounts of carbohydrate the potassium taken up by the yeast is proportional to the quantity of sugar added. Phlorizin has no effect on this reaction, sodium cyanide and sodium fluoride a small one, and iodoacetate is markedly inhib-

itory. Bakers' yeast contains 0.33 to 0.49 per cent potassium, of which 30 per cent can be washed out. According to Farmer & Jones (6) the stimulation of fermentation of glucose by potassium (0.005 per cent potassium chloride) is the same for yeast cells and for yeast extracts. The stimulation is seen most distinctly when the degradation of glucose is incomplete. A relation to the natural radioactivity of potassium is not perceptible. According to Conway (7) potassium is not necessary for growth, fermentation, or resting metabolism of yeast cells in amounts larger than 0.1 to 1.0 mg. per cent.

Experiments with radioactive potassium (K^{42}) by Hevesy & Nielson (8) show that the potassium interchange between culture media and yeast cells takes place in two hours if there is an active fermentation. In weak fermentation the potassium interchange takes place slowly. The fluctuations are not only based on differences in permeability, but are explained by various rates of synthesis of potassium compounds, which are performed under varying conditions of fermentation. According to Boyer, Lardy & Phillips (9) potassium ions affect the course of the reactions by specifically stimulating the reaction of phosphopyruvic acid with adenylphosphates. Utter & Werkman (10) did not see an accelerating effect of potassium for the attainment of the phosphoglyceromutase-enolase equilibrium.

Schwaibold & Nagel (11) found a surprisingly high zinc content (200 mg. per kg.) in yeast; the copper content was high also. In brewers' yeast Gray & Stone (12) found large variations in the iron and copper content—copper from 15 to 140 p.p.m., and iron from 40 to 200 p.p.m. A correlation between these two metals was not perceptible. Kiene (13) did not observe any effect on the rate of growth of yeast with copper, manganese, and molybdenum under exactly determined conditions, but the amount of yeast produced decreased. Iron and zinc proved to be indispensable trace elements. Raw hen's egg, able to fix iron, therefore may inhibit the growth of bacilli as well as of *S. cerevisiae* as mentioned by Schade & Caroline (14).

Selzer & Baumberger (15) analyzed the influence of metallic mercury on the respiration of yeast. The uptake of oxygen by yeast cells is inhibited completely if finely distributed mercury is stirred into a glucose solution. Mercury has no effect if the substrate is pyruvate or lactate. The inhibition is explained by the formation of a complex between sulfhydryl groups of the participating enzymes and mercury. The endogenous metabolism of yeast cells is not in-

fluenced by mercury; this is explained as a consequence of different localization of sulfhydryl groups in the yeast cell.

Uranium has a toxic effect on the cell division of yeast within the limits of 10^{-3} to 10^{-4}; concentrations of 10^{-4} to 10^{-5} have a stimulating influence (16). Yeast contains on the average 2×10^{-7} per cent uranium (17, 18). The ash of brewers' yeast which amounts to about 0.6 per cent is, therefore, radioactive.

Studies on the permeability of yeast cells to sodium fluoride have been published by Malm (19). It diffuses into the cells as undissociated hydrogen fluoride and the sodium hydroxide which is set free raises the pH of the solution. Under standardized conditions the amount of fluoride taken up by the cells is directly proportional to the concentration in the outside medium. It is difficult to wash out potassium from fresh bakers' yeast. When adding sodium fluoride, Malm (20) observed a decrease in the capacity of the cells to bind potassium. Further contributions to the question of the fluoride effect on yeast have been given by Runnstrom, Gurney & Sperber (21). They describe the differences in the fluoride sensibility of certain respiratory enzymes including enolase. Other instructions are to be found in the work of Borei (22) which also contains data about the influence of azides. According to Runnstrom (23) the time factor for the fluoride effect on respiration and fermentation is determined by the prevailing oxygen pressure. The inhibition of the fermentation is larger in yeast juice than in the living cells. With increasing amounts of phosphate the fluoride inhibition is increased in the living cells as well as in the yeast juices. The sequence in which the substrates and the minerals are combined with the yeast suspension is of importance. According to Runnstrom & Marcuse (24) the damage is greater if fluoride is added before glucose. Potassium increases the damaging effect of fluorine. This is not true for press juice; there the order of the addition is irrelevant. The conditions are similar for top and bottom yeast. According to Borei & Lindvall (25) it is practically impossible to show the effect of fluorides on fermentation and respiration at a pH above 6.2.

According to Malm (26) fresh yeast cells are permeable to phosphates. Yeast depleted by being kept in water becomes impermeable to phosphate but regains its permeability if shaken with glucose. The work of Lynen (27) demonstrates that the inorganic phosphate in growing yeast cells decreases to a very small amount when the suspension is shaken with oxygen during the periods in which the yeast

possesses a strong respiratory capacity. In the period of diminished respiratory activity the amount of inorganic and acid-soluble phosphate in the impoverished yeast cells increases. If ethanol, acetaldehyde, or acetic acid is added, the concentration of both forms of phosphate is extremely rapidly decreased and, at the same time, the respiration increases rapidly. These phenomena are perhaps related to the Pasteur effect (see p. 453).

Fink (28) contributes to the question of the minimum phosphate requirement. It is possible to cultivate torula which contains, on a dry basis, 1.5 to 2 per cent phosphorus pentoxide against 4 to 6 per cent under normal conditions. The content of the ash of this low-phosphorus yeast is but half of that of the normal torula. The protein and thiamine contents are practically undiminished. Fink & Just (29) succeeded in enriching bakers' and brewers' yeast with aneurin if thiazole and pyrimidine were added during the cultivation. The added substances were more efficient than thiamine. The biosynthetic product is located as the pyrophosphoric ester inside the cell and not in the culture medium.

Bleyer & Thies (30) report, in contradiction to the behavior of cell-free fermentation in which arsenates at low concentration produce a distinct acceleration of sugar degradation, that even small amounts of arsenate produce a distinct inhibition in living cells immediately after the addition; 97.2 per cent of the arsenate is precipitable by silver nitrate at pH 4.85, but after complete fermentation only 4.2 per cent is precipitated. This phenomenon confirms the fact that arsenate is bound in a different way in fermented solutions than in pure water. Harden (31) has also stated that arsenate, like phosphate, is esterified with sugar by enzymes, but that the arsenyl compounds are very labile and undergo immediate hydrolysis. It also explains the old statement of Buchner (32) that arsenates do not inhibit cell-free fermentation but even increase it sometimes. Later Neuberg & Leibowitz (33) demonstrated that the dephosphorylation of hexose-diphosphate gradually takes place in the presence of arsenate and that it leads to an accumulation of hexose-monophosphate, i.e., arsenate favors the elimination of one phosphoric acid group. We know today from the work of Cori and associates (34) that monophosphates are necessary for the formation of fructose-diphosphate which is again indispensable for glycolysis. Thus it is evident that fermentation takes place in the presence of arsenate without the accumulation of hexose-diphosphate. Braunstein & Levitov (35, 36) then reported that phos-

phorylation in the presence of arsenate leads to a sugar derivative in which both phosphoric acid and arsenic acid are bound in ester form; however sugar arsenate has not been isolated in definite form.

In the study of the finer processes of the arsenate effect, more knowledge about the course of the carbohydrate breakdown was found. Later, after Harden & Young (37, 38) had discovered the enormous stimulation of fermentation by fructose-1,6-diphosphate, Meyerhof, Kiessling & Schulz (39) showed that the following reaction takes place in the presence of arsenate, without any change of the reaction velocity and without any new phosphate uptake: Glyceraldehydephosphate + cozymase = 3-phosphoglyceric acid + dihydrocozymase. Warburg & Christian (40) as well as Negelein & Bromel (41) showed that glyceraldehydephosphate, in cooperation with cozymase, is able to fix phosphate and to be oxidized to 1,3-diphosphoglyceric acid. In the presence of arsenate they assumed the intermediary formation of a similar arsenylated compound: Glyceraldehydephosphate + arsenate → 1-arseno-3-phosphoglyceraldehyde. Then, in cooperation with cozymase which is simultaneously reduced to dihydrocozymase, an oxidation to 1-arseno-3-phosphoglyceric acid takes place. The latter is then supposed to decompose spontaneously to arsenate and 3-phosphoglyceric acid. Meyerhof & Junowicz-Kocholaty (42) showed that the effect, originally regarded by them as specific for the fermentation of fructose-1,6-diphosphate, concerns the acceleration of the dephosphorylation of 1,3-diphosphoglyceric acid by arsenate and is due to a reduction to glyceraldehyde-monophosphate under simultaneous splitting off of phosphate. The counterbalancing oxidation of glyceraldehydephosphate proceeds in the presence of arsenate without phosphorylation. The dephosphorylation of phosphopyruvic acid takes place in the absence of adenylpyrophosphate, but is accelerated enormously by arsenate.

Diemair & Schülke (43) describe that arsenites and arsenates are bound by fermenting yeast; this process increases with the vigor of the fermentation. An esterification of arsenate is observed when the yeast is damaged. Arsenate by itself inhibits the fermentation in living cells but stimulates their growth. According to Beraud (44) yeast can become adapted to arsenite by permanent inoculation. The adapted yeast shows morphological changes and a greater sensibility towards irradiation with ultraviolet light. Growing in arsenite-free culture media, it again acquires its normal character and multiplies faster. Pickett & Clifton (45) report that potassium arsenite inhibits.

the oxidation of glucose and pyruvic acid by yeast, that sodium azide and 2,4-dinitrophenol block carbohydrate synthesis, and that mono-iodoacetate prevents the fermentation as well as the esterification of glucose, while sodium fluoride hardly affects the oxidation of glucose at all.

Sampath (46) indicates that vanadium pentoxide in concentrations of 1 : 1000 inhibits sporulation, while sporulation is favored in concentrations of 1 : 2000. Under the influence of vanadate the vegetative cells of *S. cerevisiae* change their morphological character.

Loginova (47) has analyzed systematically the adaptation of *S. cerevisiae* to sulfur dioxide. By permanent reseeding in media with increasing concentrations of sulfur dioxide, a strain was isolated which could stand a concentration of 0.25 per cent sulfur dioxide. It fermented normally and is recommended for fermentations of fluids which are preserved with sulfur dioxide.

ORGANIC CONSTITUENTS

Tikka & Heino (48) call attention to a substance in yeast which reduces 2,6-dichlorophenol-indophenol (Tillmann's indicator for ascorbic acid). It is water-soluble, unstable against daylight, and easily oxidized by the oxygen of the air. This observation explains why fermented fruit juices occasionally show an apparently higher content of ascorbic acid than unfermented ones. The chemical nature of this yeast constituent is unknown.

The thiomethylpentose, $C_6H_{12}O_4S$, discovered by Suzuki & Mori (49) is present in yeast as adenyl-glycoside. According to Wendt (50) who applied the method of Willstatter-Schudel, the thio-sugar obtained by acid hydrolysis requires 2 mols iodine, an amount twice as much as that required by a normal aldose. After reduction with sodium amalgam, the thio-sugar yields thiopentitol. This is oxidized with exactly 1 mol iodine to the corresponding sulfoxide. The results of the oxidation with lead tetracetate show that the group CH_3S- cannot be located in position 3. The thiomethylpentose is probably to be considered as a derivative of ribose. An analogy with the adenylic acid of muscle is not unlikely.

On the basis of a color reaction between tryptophane and fructose as found by Jordan & Pryde (51), Malm (52) believes that a similar colored substance will result by drying bakers' yeast. It can be brought into solution with concentrated hydrochloric acid. From yeast, pretreated with sodium fluoride, more coloring substance can be ex-

tracted, probably as the result of an accumulation of fructose-phosphates.

It has been known for a long time that fermenting yeast is able to transform a part of the offered sugar into polysaccharides. If yeast is suspended in a phosphate solution of pH 4, according to Gottschalk (53) approximately half of the offered zymohexoses are, under anaerobic conditions, transformed into polysaccharides; from the sugar which is actually taken up *ca.* 77 per cent is recovered as carbon dioxide and alcohol. Hassid, Joslyn & McCready (54) have isolated a polysaccharide from *S. cerevisiae* by extraction with 3 per cent sodium hydroxide, 3 per cent hydrochloric acid, water, and by treatment with ethanol. This polysaccharide is nonreducing and insoluble; it is obtained in a yield of 7.9 per cent on a dry weight basis. The hydrolysis of the material $(C_6H_{10}O_5)_n$, containing 0.56 per cent nitrogen, with concentrated hydrochloric acid, yields glucose in the form of β-glucose. 2,4,6-Trimethylglucose was obtained by the usual methods, but no tetramethyl derivative was found. The authors came to the conclusion that the polysaccharide belongs to the closed chain type. Glycogen from bakers' yeast has been reinvestigated by Teauloz (55). It is similar to animal glycogen, but the phosphorus content is higher.

Barry & Dillon (56) have extracted a polysaccharide from yeast by treatment with diluted alkali. It is said to be a 1,3-glucan. It contains one end group for each 28 glucose units. By oxidation with periodic acid a substance was isolated which, after hydrolysis, gave an osazone; it was considered identical with laminaridiosazon. The hydrolysis of this glucan with hydrochloric acid yields oligosaccharides which are decomposed by emulsin, but not by takadiastase. Accordingly, a β-configuration is attributed to the glucan by which it differs from laminarin. Garzully-Janke (57) gives an improved method (exact details and modification of Salkowski's old method described in 1894) of the isolation of mannan from yeast. Yeast press juice and rapidly prepared yeast autolyzates do not contain mannan. Haworth, Heath & Peat (58) discuss the probable structure of mannan from bakers' yeast on the basis of the fact that the polysaccharide is homogeneous. The methods developed by Haworth and his school (exhaustive methylation and hydrolysis) show that 3,4-di-, a mixture of 2,4,6-, and 3,4,6-tri-, and 2,3,4,6-tetramethyl mannose are formed in equimolecular proportions. A small quantity of 2,3,4-trimethylmannose (3 per cent of the methylated mannan) is present in addition.

The mannan consists entirely of d-mannopyranose residues, joined by α-glycosidic linkages.

Woolley (59) has determined the content of inositol in brewers' yeast to be 5γ per mg.

By extraction with a mixture of 80 parts of benzene and 20 parts of ethanol, 4.07 per cent fat and 0.19 per cent phosphatides are obtained from vinegar yeast according to Rewald (60). With the same treatment brewers' yeast yields 0.93 per cent fat and 0.78 per cent phosphatides. Dirr & von Soden (61) found that Bergin yeast contains 4.5 per cent of its dry weight as phospholipids. The contents of lipids found in yeast depends *in toto* to a large extent on the way it is grown.

For the zymosterol from yeast Wieland, Rath & Benend (62) have established the empirical formula $C_{27}H_{43} \cdot OH$. One of the existing double bonds can be reduced catalytically. No acetone is obtained from this dihydrozymosterol by ozonolysis, but it is obtained from the original compound. From this it may be concluded that the easily reducible double bond is located in the side chain. After rearrangement with hydrochloric acid, the second double bond can also be reduced; thereby the well known cholestanol is formed. The dihydrozymocholesterol is not identical with any of the four known isomeric cholestanols. The zymosterol can be converted into zymostenon as well as into α- and β-zymostenol ($C_{27}H_{46}O$) and into zymostadienone. The pure zymosterol melts at 108–110°, $[\alpha]_D = +49°$ in chloroform. Wieland, Rath & Hesse (63) have isolated from yeast ascosterol, $C_{29}H_{45} \cdot OH$, as well as fecosterol, episterol, and neosterol; the latter is perhaps identical with isoergosterol. It contains three double bonds, one of which is located in the side chain and two in the nuclei. Besides ergosterol and the other sterols mentioned above, Wieland & Joost (64) have isolated a new substance, cryptosterol. With dry hydrogen chloride it is converted into the hydrochloride $C_{30}H_{52}O_2$. With silver oxide cryptostenediol, $C_{30}H_{52}O_2$, is formed from it. This can be oxidized with chromium trioxide to cryptostanedione, $C_{30}H_{48}O_2$. After conversion to the hydrazone, $C_{30}H_{48}N_2$, the triketone $C_{30}H_{46}O_3$ can be isolated. By distillation of dihydrocryptosterol with boron oxide the hydrocarbon $C_{30}H_{50}$ is obtained. The constitution of the cryptosterol and the lanosterol, also extracted from yeast, has not yet been clarified completely (65). Secondary sterols are in question. It is remarkable that the acetate of lanosterol, treated with osmium tetroxide, yields a stable ester of osmic acid.

Further details of zymosterol and its transformation products are given by Wieland & Benend (66).

The formation of ergosterol depends on the oxidizing activity of the yeast cells. If cultivated anaerobically the yeast contains 0.2 to 0.3 per cent ergosterol; the amount is usually increased by aeration (67). According to Zorkoczy (68), an increase of ergosterol can be obtained up to three to four times that of normal yeast, by the fatty degeneration of yeast produced by aeration and exclusive nutrition with ethanol and acetaldehyde. According to a patent granted Bennett (69), an increased ergosterol content is obtained by cultivation of yeast in the presence of nontoxic oxidizing agents (persulfate, peracetate, and other peroxides) and subsequent aeration in a nutritive medium deficient in assimilable nitrogen.

According to statements by Myrbäck et al. (70) the thiamine-disulfide, described by Zima & Williams (71), can be found in bakers' yeast. Schönberg & Sperber (72) do not believe in the evidence of this statement. They show that the principal part of the substance is cocarboxylase and that it may also perhaps contain some thiamine. They state the remarkable fact that the phosphatases of yeast are not entirely inactivated by boiling for one or two minutes in water or $0.1 N$ hydrochloric acid. This resistant phosphatase furnishes a detectable amount of free thiamine.

Rimington (73, 74) has established the formation of porphyrin by autolyzing yeast and yeast press juice. The chemical reaction for the formation of porphyrin by autolyzing yeast apparently takes place in a cell-free medium. A suitable yeast must be chosen. After pressing, a fractionation is performed and a stable active material which can be stored is produced. Thus, a thermostable porphyrin precursor or the raw material for porphyrin synthesis was found in the concentrate. Green et al. (75) have isolated a new flavoprotein from top yeast. A yield of 40 mg. of the substance containing 344γ of flavine-adenine-dinucleotide was obtained from 500 gm. of dried yeast. When the solution of the chromoprotein is boiled or acidified the flavine group is split off and the protein itself is denatured. With sodium sulfate the color is bleached to 46 per cent. The nonflavine colored group is not reducible with sodium sulfate and is not detached from the protein when the flavoprotein is denatured. The flavoprotein contains at least 1.4 per cent of flavinphosphate. This is more than twice the percentage found in typical flavoproteins. A catalytic function of the chromoprotein has not yet been established. It is not identical with

any of the known flavoproteins. It is the substance or one of the substances which cause the yellowish color of the maceration juices of brewers' yeast.

After cytolysis of yeast with ether Kazakov (76) obtained proteins by aqueous extraction. By heating to 80° a thermo-unstable protein part can be separated. A thermostable fraction is obtained on concentration of the filtrate. According to another method, dried yeast is extracted with water and again a protein is precipitated by heating to 80°. In addition to the usual amino acids, purines are formed by hydrolysis (undoubtedly originating from yeast-nucleic acids).

The secondary changes which the amino acids undergo during the hydrolysis of proteins—especially in the presence of other substances and with respect to the sulfur-containing components have been studied by Grossfeld & Young-Yen (77). It was concluded that one part of the sulfur, originally contained in the organic material, escapes in the form of volatile compounds or is converted into sulfate during the hydrolysis.

Block & Bolling (78) give data for the essential amino acids (arginine, histidine, lysine, tyrosine, tryptophane, phenylalanine, cysteine, methionine, threonine, leucine, isoleucine, valine) and the total nitrogen and sulfur content of yeast. The protein of the *S. cerevisiae,* grown on different culture media, shows a quite constant content of ordinary amino acids. On the other hand, a variation in the composition of the yeast proteins is reported by Luers & Vaidya (79), if the nitrogen content of the nutrient solution (molasses) is decreased to an abnormally low value. The most prominent change concerns the arginine which, in terms of percentage of total nitrogen, falls from 3.5 to 0.5 and the histidine, which rises from 2.7 to 6.5. These comparative experiments have been made with normal compressed yeast and with yeast grown under aeration in a weak solution of molasses. According to Ryan & Brand (80) the microbiological method, using the leucineless strain of *Neurospora crassa,* can be applied to the determination of the leucine content of hydrolyzates of yeast.

In view of the importance of *Torulopsis utilis* as fodder yeast, its metabolism is well worthy of attention. Lechner (81) establishes again that the micro-organism can utilize zylose almost as well as glucose. Fink & Just (82) demonstrated that it contains ducitol when it has been grown in wood sugar solution. It is not certain whether this is formed by reduction from the galactose of the culture medium

as an accidental constituent or whether it has a part in metabolism similar to that of trehalose in bakers' yeast (83). Sperber (84) constructed a respirometer for exact measurement of the gas metabolism of *Torula;* the yeast suspension is placed in a Kluyver flask. In this apparatus all sorts of yeasts can be grown under anaerobic conditions, giving yields that equal those obtained on an industrial scale. As for the attack of *Torulopsis* on nitrogen-containing substances (ammonia, acetamide, α- and β-alanine, asparagine, urea, guanidine, several aliphatic primary and secondary amines, benzylamine, the common amino acids, nitrites, and nitrates), a distinct difference can be observed between yeasts with a high nitrogen content and those containing a low percentage of nitrogen. In the course of experiments lasting for three or five hours the former do not utilize the compounds named, while the latter do. Except in the presence of ammonia, urea, guanidine, methylamine, glycine, asparagine, α-alanine, and propylamine, a simultaneous respiration of glucose is, however, required. For both types of *Torulopsis* the composition of protein, carbohydrates, lipids, and ash was reported and except for ash a very distinct difference was found.

Lindegren (85) has investigated the part played by fat and carbohydrate storage in the induction of dormancy in yeast cells and the conditions of reactivation. The fact that dormant cells require a medium containing a relatively full complement of nutrients enables the cells to start growing under conditions that assure continued growth. The fact that they require specific vitamins which they are unable to synthesize is important for the general problem of inducing fungal spores to germinate.

Since excellent recent reviews (86 to 94) are available on general carbohydrate metabolism, oxidation-reductions, the biochemistry of fungi, the chemistry and metabolism of the compounds of phosphorus, co-enzymes, etc., only the latest results or those to which no consideration has been given previously need be mentioned.

Enzymes and their effects.—For the process of anaerobic alcoholic fermentation yeast must provide 20 or more enzyme proteins and at least three, possibly eight, dissociable organic co-enzymes, as well as several inorganic catalysts (ammonium, potassium, magnesium, manganese, copper). Thus twelve stable intermediary stages and the end products, carbon dioxide and ethanol, are produced.

Myrbäck & Vasseur (95) studied the fermentation of lactose by *S. fragilis* and *Torula cremoris* to determine the localization of en-

zymes. Under varied conditions both these yeasts ferment lactose
faster than a mixture of glucose and galactose. The authors draw the
conclusion that the disaccharide is fermented by a lactozymase, located
inside the cell, while the hydrolyzing enzyme lactase is located on the
outer side of the membrane. The rapidly diffusing lactose is fer-
mented without previous hydrolysis.

The typical degradation of the zymohexoses to 3-carbon deriva-
tives, formerly regarded as an effect of glycolase, is now considered
due to the presence of the unstable furanose derivative, fructose-1,6-
diphosphate. The other hexosephosphates are pyranose derivatives;
hence the following facts are remarkable.

The formation of a furanose modification is an essential phase of
fermentation processes.

Yeast has no or at least not a distinct selective ability toward the
cis-trans (α-resp. β-) forms of the zymohexoses. This does not apply
if the ring specificity is taken into consideration. The first worker to
make this scarcely noticed observation was Pringsheim (96). The
heteroglucose (stable γ-glucose) obtained by him and in which a
7-carbon ring has been taken for granted, can very easily be trans-
formed into normal glucose by the addition of dilute hydrochloric
acid. It is, however, not fermentable. All sugars with the exception
of crystallized lactulose as stated by Pigman, Frush & Isbell (97, 101)
are pyranoses in the solid form (98). When they are dissolved in
water they change with different velocity into equilibrium mixtures
of pyranose and furanose. Hopkins & Roberts (99) observed that
the speed of fermentation at lower temperature is much slower for a
freshly prepared solution of crystallized pyranoide *d*-fructose than
for an old equilibrium mixture of fructo-pyranose and furanose and
that the temperature coefficient of the fermentation is the same as that
of the mutarotation. According to Hudson (100) as well as Isbell &
Pigman (101) this is the indicator for the pyranose-furanose inter-
conversion. The results of Hopkins & Roberts are confirmed by
Gottschalk (102). He demonstrated that at 0° and pH 4.3 *d*-fructo-
β-pyranose is fermented at a decreased rate compared to α-*d*-glucose,
which is also fermented in its furanose modification. Lehmann &
Needham (103) show the importance of the ring specificity for the
glycolytic processes. The dimeric ring modification of glyceraldehyde
inhibits phosphorolysis of glycogen; the monomeric glyceraldehyde
is an inhibitor of glycolysis.

Special fermentations.—Neuberg & Hofmann (104) demonstrated

that apart from the zymohexoses both trioses can be fermented—at least with certain yeasts. This is possible far more frequently than previously suggested and without any special relation to the 3-carbon sugars.

It is significant that quite different substrates are also fermented. 5-Keto-d-fructose (5-d-fructonese),

$$
\begin{array}{c}
 H \quad\ OH \\
 | \qquad | \\
CH_2OH \cdot CO \cdot C \cdot C \cdot CO \cdot CH_2OH \quad (105) \\
 | \qquad | \\
 OH \quad H
\end{array}
$$
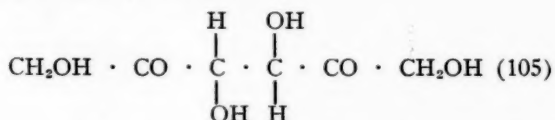
is fermented by top yeast, yielding 1 mol carbon dioxide. The isomeric 5-keto-d-glucose,

$$
\begin{array}{c}
 OH \quad H \quad\ OH \\
 | \qquad | \qquad | \\
COH \cdot C \cdot C \cdot C \cdot CO \cdot CH_2OH \\
 | \qquad | \qquad | \\
 H \quad\ OH \quad H
\end{array}
$$
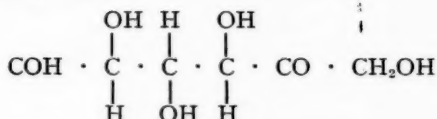
is not fermentable. Similarly, the methyl tetrose phosphate,

$$
CH_3 \cdot CHOH \cdot CHOH \cdot CO \cdot CH_2 \cdot O \cdot PO_3H_2,
$$
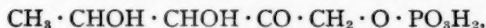
obtained by Meyerhof, Lohmann & Schuster (106) by condensation of triose phosphate and acetaldehyde is fermented by yeast juice. It dissociates into its components and the triose phosphate ferments in the usual way. The free methyl tetrose is not fermentable. Warburg, Christian & Griese (107) believed that 6-phosphogluconic acid could be fermented, but later did not insist on it (108). Lipmann (109, 110) also made some remarks about the fermentability of phosphogluconic acid, but did not follow them up.

Dickens (111) has isolated an enzyme from the maceration extract of bottom yeast, which oxidizes phosphogluconic to phosphoketo-gluconic acid and a phosphopentonic acid. A phosphoerythronic acid may also be formed. The author's statement that d-ribose-5-phosphoric acid is completely fermented by dried brewers' yeast and by maceration extract is especially important. One mol each of phosphoric acid, ethanol, carbon dioxide, and an as yet unidentified product are formed. Since there is no reducing action glycollicaldehyde cannot be present. Dickens believes that dismutation to ethylene glycol and glycollic acid takes place. d-Arabinose and d-xylose phosphoric acid are fermented to a lesser degree. In this connection it is

interesting to note that Goepfert & Nord (112) were able to obtain glycollicaldehyde by the degradation of ethylene glycol with fusaria.

Isbell (113) has shown that d-talose, which he obtained in pure condition is not fermented by yeast; the latter cannot be adapted to this sugar either.

Fermentation to glycerol.—The production of glycerol by fermentation of sugar has been of importance in both world wars. The well known basis of the second type of fermentation,

$$C_6H_{12}O_6 = CO_2 + CH_3 \cdot CHO + CH_2OH \cdot CHOH \cdot CH_2OH,$$
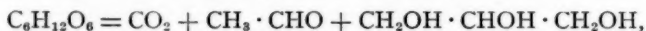
is the fixation of the acetaldehyde by addition of soluble or insoluble sulfites. By the elimination of this oxidation phase from the final reduction to ethanol, glycerol accumulates as a correlative reduction effect. According to previous experiments the zymohexoses behave in the same way. Under comparable conditions Lees (114) found that the fermentation of maltose took five times as long. According to Hickey (115) free ammonium hydroxide proves to be a very toxic agent in the process. Lechner (116) gave a detailed and generally correct description of the process and the particulars involved. A new prospect is opened by a patent granted to Haehn (117). This author applied the much used method of producing yeast by aeration to the sulfite process with the objective of shortening the longer lasting fermentation time of from three to six days to eight to ten hours and claims that the yield of glycerol was increased. Both these claims are of considerable importance economically. A thorough investigation of the problem by Neuberg & Maengvyn-Davies (118) led to the following results, which have not yet been published. The authors were unable to complete the sulfite fermentation in eight to ten hours, but needed fourteen to eighteen hours. The beneficial action of aeration is due to the improved state of the yeast under the unfavorable conditions in the sulfite mash. Oxidation of the sulfite is relatively insignificant. Similar conditions were found with nitrogen; namely that swirling of the mash by the injection of gas has a beneficial effect. Previously it was generally accepted that glycerol fermentation could be accomplished only with top yeast. Neuberg & Maengvyn-Davies demonstrated that brewers' yeast can be used with almost identical results. If the sulfite concentration is the same, approximately the same yield of glycerol is obtained both with and without aeration. Temperature variations have no decisive influence. It has been known for a long time that trimethylene glycol can be formed when sugar is

fermented to glycerol. It is produced by a secondary reaction of bacteria on the glycerol. A study of Mickelson & Werkmann (119) further clarifies this process.

Views put forward by Nickerson & Carroll (120) are of interest. They state that the action of *Zygosaccharomyces acidificans* on sugar causes the so-called third type of fermentation, i.e., large accumulation of acetic acid and glycerol, in addition to ethanol.

Herbert, Gordon, Subrahmanyan & Green (121) prepared highly enriched zymohexase in the form of a white feathery powder. It is sensitive towards organic solvents, but relatively stable when dry or at 0°. It easily dissolves in water to a colorless and relatively stable solution. Even in small traces heavy metals cause considerable inactivation of the enzyme. Of the sugars and sugar phosphates studied fructose-6-phosphate has the strongest competitive inhibition effect on the scission power of the enzyme. α-Glycerophosphate does not act as an inhibitor.

By the method of Neuberg & Lustig (122) fructose-1,6-diphosphate can be prepared from bakers' yeast. The advantages of this process are that the washing and pressing needed in the utilization of brewers' yeast are eliminated. Bakers' yeast can be used immediately without any drying. The following types of yeast can be used: Atlantic, Federal, Blue Ribbon, and National Grain. Fleischmann's yeast phosphorylizes well after having been dried at room temperature.

The neutral, alkaline-earth salts of fructose-1,6-diphosphoric acid, e.g., $C_6H_{10}O_4(PO_4Ba)_2$, are difficultly soluble in water. Soluble alkaline earth salts are preferable as substrates. Neuberg, Lustig & Rothenberg (123) describe the behavior of the easily soluble dibasic alkaline earth salts, e.g., the compound $[C_6H_{10}O_4(PO_4H)]_2Ba$. The completely pure fructose diphosphoric acid has a rotation $[\alpha]_D^{17} = -4.04°$ to $4.15°$, while formerly $[\alpha]_D^{15}$ was found to be $+3.55°$. The power of reduction is 48 per cent of pure fructose. If bromine water is used isomerizations as in the hypoiodite method are eliminated; the pure substance does not use any halogen and is therefore free of aldose components. Tribasic alkaline earth salts are also known, e.g., $(BaO_4P \cdot C_6H_{10}O_4 \cdot PO_4H)_2Ba$. They are also easily soluble in water.

The same authors (123) also improved the method of preparation of *d*-fructose-6-phosphate. By low temperature hydrolysis they removed the phosphoric acid group located in position 1. The barium salt of *d*-fructose-6-phosphoric acid, which they obtained in great

purity, had a rotation $[\alpha]_D^{19} = +3.58°$. The reducing power is 82 per cent that of d-fructose. It does not include any aldose derivative. McCready & Hassid (124) describe the purification of glucose-1-phosphate by means of ion exchange. The method can be applied to other hexose phosphates.

Wolfrom & Pletcher (125) and Wolfrom, Pletcher, Smith & Brown (126) successfully synthesized the ester of Cori, Colowick & Cork (127), thus finally proving that it is the α form of d-gluco-pyranose-1-phosphate.

In 1937 Kosterlitz (128) astonished biochemists by the discovery of an unknown galactose monophosphoric acid. By its synthesis (129) this has been recognized as galactopyranose-1-monophosphate. It forms a well-crystallized dipotassium salt, $C_6H_{11}O_9PK_2$, $[\alpha]_D^{18} = +108.0°$. It is resistant to alkali, not reducing, and is completely hydrolyzed by $0.09 N$ sulfuric acid at $100°$ in two minutes. It is a stronger acid than phosphoric acid (130). By the phosphatase of $S.$ $ludwigii$ it is split into its components; living and dried galactose-adapted $S.$ $cerevisiae$ F. ferment it. The galactose-1-phosphate is an intermediary in the fermentation of galactose by galactose-adapted yeast (131). With the juice of the latter it is fermented in a characteristic manner, similarly to the naturally occurring esters of Cori, Neuberg & Robison.

Experiments which have not yet been published show that the results obtained by Smythe (132) can be reproduced, provided that his method is followed absolutely. The α-double salts of glucose-6-phosphate and α-glycerophosphate described by him are important because of their ability to crystallize. The components are present in a stoichiometric proportion which Meyerhof, Kiessling & Schulz (133) failed to find.

Kalckar (134) mentions that d-galactose and l-arabinose can be phosphorylated.

The transformation of fructose-6-phosphate into fructose-1,6-diphosphate by means of adenosinetriphosphate was accomplished ten years ago (135) by the Parnas reaction, the transphosphorylation of heterogeneous compounds. In this experiment the phosphorylation by living yeast through the isolation of the reaction product was made evident for the first time. Today this reversible process is inserted as an indispensible reaction in the scheme of glycolytic processes. Regarding its formulation see also Kalckar (134), Doudoroff (136), Potter (137), and Colowick & Price (138).

By the new method of Neuberg & Lustig (139) which is an improvement of the 1933 process of Neuberg & Kobel, $d(-)$3-phosphoglyceric acid can now be prepared conveniently from bakers' yeast. Statements (140) that bakers' yeast does not accumulate phosphoglyceric acid like brewers' yeast are incorrect. The acetaldehyde used as hydrogen acceptor heretofore can be suitably replaced by furfural in this process. Instead of starting with the comparatively expensive fructose diphosphate, it is now possible to proceed directly from the crude phosphorylation mixture. Schlenk & Schlenk (141) show that $d(-)$3-phosphoglyceric acid can replace the difficulty accessible phosphopyruvic acid in the identification of adenosine-5-phosphate and its homologues.

With the optically active $d(-)$glyceric acid, accessible according to Neuberg & Lewy (142) as the starting material, $d(-)$3-phosphoglyceric acid and $d(+)$2-phosphoglyceric acid could be synthesized. The synthetic products are identical with the natural ones in every respect. Both Warburg & Christian (143) and Negelein & Brömel (144) postulated the formation of d-glyceraldehyde-1,3-diphosphate as an intermediary phase of the reaction: d-glyceraldehyde-3-phosphate $+$ phosphate $+$ cozymase $=$ dihydrocozymase $+$ d-glyceric acid-1,3-diphosphate. Drabkin & Meyerhof (145) come to the conclusion that the phosphorylation is brought about by direct phosphorylating oxidation. Spectrophotometrical investigation favors the view that an unstable addition product is the precursor of glyceric acid 1,3-diphosphate. The synthetic (dimeric) glyceraldehyde diphosphate of Baer & Fischer (146) cannot be considered as this precursor.

Nilsson (147) has made a series of objections against the validity and applicability of the Embden-Meyerhof scheme to explain glycolysis. Essentially his objections are based on the fact that intact dried yeast does not show the same break in fermentation rate as do preparations of normal fermenting yeast after half the sugar is fermented. According to Nilsson this break is due to the fission of hexose in a phosphorylated and an unphosphorylated half. In living cells both compounds are fermented with equal speed, but once the cell organization is destroyed only the phosphorus-free half is fermented, while the phosphorylated half condenses to fructose diphosphate by a process which does not exist in nature. Meyerhof (148) counters these views and similar ones put forward by Ivanov in 1909 (149), von Euler in 1911 (150), Kluyver & Struyk in 1928 (151), and Dische in 1933 (152) by the following contentions: The two phases

of cell-free fermentation are a result of the destruction of the sensitive and structurally bound adenylpyrophosphatase (apyrase) of living yeast during drying and extraction. When concentrated adenylpyrophosphatase is added, the cell-free fermentation of hexosediphosphate is increased to the maximum rate of sugar fermentation. The adenylpyrophosphatase of yeast can be brought into solution only with great difficulty; the best method is supersonic vibration. It is highly unstable, even when kept in the refrigerator. Addition of glutathione or treatment with ethanol and aluminum hydroxide, which eliminates part of the proteins, improves the stability of the preparation. It must be mentioned that the optimum pH is at 9, outside the range of other fermentation enzymes. Enzyme activity is increased by manganese ion but not by magnesium, calcium depresses activity, potassium cyanide is ineffective, and sodium azide has a strongly inhibiting effect.

As for the question whether phosphorylation must be regarded as an essential phase of the transformation of sugar yeast, Nord & Mull (153) have a reserved point of view.

Pigman (154) proved that complete transformation to fully fermentable carbohydrate takes place when starch is hydrolyzed by amylase in the presence of yeast. This demonstration is of both practical and theoretical importance. If the yeast is added only after reaction of the amylase this transformation does not occur; in this case the amylases of maltose synthesize unfermentable carbohydrate. Schultz, Atkin & Frey (155) showed that the rate of fermentation of galactose and maltose by yeast is higher in pure oxygen, at a pH of 5.4, and with constant shaking than under normal conditions.

Van Niel & Anderson (156) contribute to the question of how much sugar can enter into actual fermentation. They find that 70 per cent is fermented by *S. cerevisiae,* 30 per cent being used for "fermentative assimilation." Together with Cohen, van Niel (157) studied the phenomenon of fermentative assimilation with *Candida albicans.* They show that this assimilation is most pronounced with young cells in glucose solution. The aerobic decomposition of glucose can be expressed by the formula $C_6H_{12}O_6 + 2O_2 = 2H_2O + 2CO_2 + 4(CH_2O)$; that of ethanol by $C_2H_5OH + O_2 = 2H_2O + CO_2 + CH_2O$; that of acetic acid by $CH_3COOH + O_2 = H_2O + CO_2 + CH_2O$; that of pyruvate by $2CH_3CO \cdot COOH + 3O_2 = 2H_2O + 4CO_2 + 2(CH_2O)$. (In all these formulas CH_2O is the expression denoting the assimilation product).

Musfeld (158) made exact measurements of the absorption of sugar by glycogen-free and dormant yeast cells. The uptake of glucose is about seven times larger than that which the same quantity of yeast would utilize for fermentation and respiration at the same temperature and in the same time. The greater part of the absorbed sugar is transformed into glycogen. Pickett & Clifton (159) found that about one third of the sugar consumed by oxidation and respiration is present in the form of fat, glycerol, fructose diphosphate, acetic acid, pyruvic acid, and hydroxy acids.

The ultimate reason for the Pasteur effect has not yet been definitely established [see Lipmann (90)]. Johnson (160) believes that the explanation can be found in the fact that the phosphoric acid liberated from phosphoric acid compounds by energy consuming processes can be re-esterified by a lower rate of carbohydrate utilization under areobic conditions. In anaerobic processes less phosphoric acid is consumed and fewer energy-rich phosphoric acid compounds are formed. The author therefore suggests that oxidative phosphorylation is a measure and a condition of the Pasteur effect. According to Engelhardt & Sakov (161) this process occurs with the Neuberg ester. The phosphorase of this ester is very sensitive toward oxidation and is completely inhibited if the oxidation-reduction potential is higher than 0.05 v. During aerobiosis neither the fermentation of fructose diphosphate nor the reaction between glucose-6-phosphate and fructose-1,6-phosphate, nor the isomerization of glucose-6-phosphate to fructose-6-phosphate is effected. The authors therefore conclude that the Pasteur effect is related to the transphosphorylation reaction of fructose-6-phosphate and the adenyl system. Gottschalk (162) arrives at entirely different conclusions. Under anaerobic conditions, in the completely compensated system, the dihydrocozymase is dehydrogenated by the hydrogen acceptors normally formed during the degradation of sugar. Under aerobic conditions the dihydrocozymase is only partially reoxidized by oxygen, i.e., by means of the diaphorese-cytochrome-oxidase system. The part which remains in its reduced form is not available for glycolytic processes, so that part of the carbohydrate is saved from degradation. As an illustration of this theory the author (163) mentions the process of phytochemical reduction of diacetyl, discussed on page 464. Melnick (164) attributes the occurrence of the Pasteur reaction to a special enzyme. He regards it as different from the ordinary respiratory enzyme, which causes the oxidation of the products of metabolism. The CO-com-

pounds are similar and show the absorption spectrum of the pheo-hemin protein; the Pasteur enzyme, however, lacks the band at 560 mμ.

In general, bakers' yeasts cannot be utilized for the preparation of active zymase extracts. Neuberg & Lustig (165) demonstrate that after being dried, commercially available bakers' yeast can be so used. The extraction can be performed at 37°, at room temperature, or at +4°, but must be made with diammonium phosphate, for 300 gm. dried yeast with a solution of 22 gm. diammonium phosphate in 1000 cc. tap water. During this process the pH decreases from 8.3 to 6.6. The coagulable protein content of the clear juices is high, 2.6 to 3.3 per cent, i.e., three times more than in the case of water extraction. It is possible that this as well as the presence of ammonium ions accounts for their desirable properties. Neuberg & Schwenk and Harden *et al.* recognized a long time ago that ammonium ions are indispensable for some partial processes of fermentation. Active zymase can still be extracted from the dried yeast after the third diammonium phosphate extraction. It is therefore impossible that the release of active zymase could be due to osmotic action alone.

To prepare apozymase from bakers' yeast Govier (166) recom-mends addition of 25 cc. carbon tetrachloride to a suspension of 100 gm. yeast in 1.5 l. of water, stirring of the suspension for an hour, centrifuging, and drying of the residue. The residue is again suspended in 21. water, treated as before, and dried in a stream of air.

Nachaeva (167) investigated the proteolytic decomposition dur-ing the drying of yeast. Because of the many uses of dried yeast these processes are noteworthy. If the yeast is dried at 45° carbon dioxide is liberated; even at 10 to 15° proteolysis takes place. The proteolytic decomposition of the yeast proteins is diminished by potassium bromate, silver nitrate, and potassium manganate in the indicated sequence and in amounts of 0.01 per cent, calculated on the water content of the compressed yeast.

Even though carbon dioxide is an obvious end product of yeast metabolism, its metabolic effect has not been studied very much. The discovery of Wieland and co-workers (168, 168a, 168b) that yeast is able to fix carbon dioxide has not been given sufficient attention. Brandt (169) makes an extensive study of this phenomenon and ar-rives at new results. He found, for instance, that carbon dioxide has no influence under anaerobic conditions on cell metabolism, rate of absorption, fermentation of glucose, or on the pH. The free phosphate

content of the cells remains unchanged. Under aerobic conditions carbon dioxide has a distinctly inhibitory effect upon the growth of yeast. Carbon dioxide activates endogenous respiration and the absorption of glucose and pyruvate. The free phosphate content of the cell diminishes, but phosphoric acid does not appear in the suspension medium. The pH also changes. Under anaerobic conditions the carbon dioxide is acid labile and is bound, possibly as bicarbonate; in the presence of oxygen it is bound in some other manner, only a small percentage being present as carbamate.

Parve (170) made some instructive experiments with carboxylase. Apozymase combines with cocarboxylase and magnesium totally and rapidly at a pH of 6.8. The reconstituted enzyme system ferments pyruvic acid at an optimum pH of 5.6, just like natural carboxylase.

The oxidation of pyruvic acid was reinvestigated by Chen & Tang (171). Cyanide and methylene blue affect oxygen uptake and carbon dioxide production. Since both agents have no effect on the oxidation of acetaldehyde, it is assumed that the oxidation of pyruvate is a direct one. By means of α-keto glutaric acid Ochoa (172, 173) demonstrated conclusively that decarboxylation can be coupled with phosphorylation. Von Euler & Högberg (74) supply information about the amount of carbon dioxide evolution of sodium pyruvate with changing pH (6.2 to 4.5), in acetate buffer, and with varied content of apo- and co-zymase.

Haag & Dalphin (175) claim that the accumulation of pyruvic acid during alcoholic fermentation in a synthetic medium depends on the cocarboxylase content and that this has a greater effect than a decrease in pH. If the carboxylase concentration falls below 3×10^{-9} per cent pyruvic acid is not decarboxylated.

New investigations on the formation of fusel oil have been made by Dietrich & Klammerth (176). Fusel oil forms chiefly at the beginning of the fermentation. If no sugar is present yeast does not form any amyl alcohol from leucine. When yeast becomes poor in reserve material as a result of previous shaking with oxygen, it produces increased amounts of fusel oil. Addition of charcoal is said to increase the yield of fusel oil.

Hohl (177) states that small amounts of formic acid are found in grape juice. In the presence of sugar this acid is consumed faster than it is formed. It is supposed to be formed from pyruvate, dihydroxyacetone, peptone, and tannin at a pH of 2.5.

According to Peynaud (178) fermenting yeast reduces added

volatile fatty acids and acids formed in the early stages of fermentation to the corresponding alcohols. Cysteine increases the reduction effect. During the fermentation of grape juices such a process occurs naturally; the final acetic acid content depends on the balance between the dismutation of acetaldehyde and the reduction of the formed acetic acid. It differs according to the medium and to the strain of yeast (see p. 464).

Lynen (179) made an extensive investigation of the decomposition of acetic acid by yeast. He finds that first of all oxaloacetic acid is produced from the pyruvate; this condenses with acetic acid to form citric acid. This condensation takes place whenever an aldehyde, e.g., acetaldehyde, is dehydrogenated to an acid simultaneously. Succinic acid is transformed to oxaloacetic acid by way of fumaric and malic acid. It then condenses with acetic acid to form citric acid. By way of α-keto glutaric acid this is decomposed to β-aldehydo propionic acid, which again yields succinic acid. According to its concentration malonic acid inhibits the dehydrogenation of succinic acid completely or considerably and thus affects the degradation of acetic acid. If no oxaloacetic acid is available, succinic acid cannot be transformed into fumaric acid and the acetic acid cycle is interrupted. By adding fumaric acid it can be restored.

Kleinzeller (180) found that the formation of succinic acid in suspensions of resting yeasts (brewers', bakers', *Torulopsis utilis*) is proportional to the uptake of sugar. Up to 4 mols succinic acid may be accumulated for 100 mols of fermented sugar. Since the quantity is more than the dry weight of the yeast, it cannot originate from the yeast proteins. Additions of malic acid, oxaloacetic acid, aspartic acid, α-keto and hydroxy glutaric acid increase the succinic acid yield. (This view may be compared with the reflections of Rubner & Ehrlich who forty years ago considered a cycle of composition and decomposition of yeast protein.)

In 1925 Haehn & Kintoff (181) started to explain the mechanism of fat formation by yeasts. They found that acetaldehyde can be isolated by means of the sulfite trapping method when synthesis of fat by *Endomyces vernalis* is effected and that acetaldehyde is a useful substrate for biological synthesis. Reichel & Schmid (182) continued the investigation and observed that unsaturated higher aldehydes formed by condensation of acetaldehyde constitute the next intermediary stage. They made use of the same yeast. Nilsson *et al.* (183) studied the formation of fat by different yeasts. For large scale

production *Rhodatorula glutinis* was found to be the best fat former. The fat content of the dried yeast could be raised to 35.4 per cent.

Tauson (184) reports that several yeasts are able to utilize paraffine. Carbohydrates, bios, and vitamins are formed by the degradation of paraffine via fatty acids.

According to Marcuse (185) old or somewhat inactivated maceration juice inhibits fermentation in the same way as phosphates. Phosphate inhibits the formation of phosphoglyceric acid and the decarboxylation of pyruvate, but it equally inhibits the whole process of alcoholic fermentation. By previous addition of acetaldehyde the inhibiting effect of the phosphate on the formation of phosphoglyceric acid is eliminated. The point of attack of the phosphate is believed to lie between the phosphoglyceric acid and the pyruvate (186).

Stacey (187) demonstrated that remarkable amounts of dextran are produced within forty hours by *S. cerevisiae* in symbiotic association with *Leuconostoc mesenteroides,* whereas with *L. mesenteroides* alone 240 hours are required for the formation of similar amounts of dextran.

In view of the fact that *p*-aminobenzoic acid inhibits the bacteriostatic effect of sulfanilamide, the discovery of *p*-aminobenzoic acid in yeast must be mentioned. Woods (188) and Rubbo & Gillespie (189) have shown it to be present, and Blanchard (190) proved that yeast contains free as well as combined *p*-aminobenzoic acid. The latter is a growth factor for *Clostridium acetobutylium.* Before this growth factor was known Weizmann & Rosenfeld (191) had already observed that its yield can be increased by autolysis of yeast. It is isolated after previous plasmolysis and autolysis of the yeast. Commercial pressed bakers' yeast contains 7.9 mg. per 1000 gm. Ratner *et al.* (192) showed that the bound *p*-aminobenzoic acid is present in the form of a polypeptide. It is definitely acid, soluble in water and ethanol, and can be dialysed. The polypeptide apparently consists of 10 to 12 glutamic acid residues in a chain linked with the carboxyl group of *p*-aminobenzoic acid. The amino group of the latter is free. Lampen, Baldwin & Peterson (193) showed that *p*-aminobenzoic acid is formed under the most varied conditions during the growth of yeast. It may be mentioned here that according to Auhagen (194) *p*-aminobenzoyl-1-glutamic acid is ten times as strong an antagonist of *p*-sulfanilamide as *p*-aminobenzoic acid. This is true only for the peptide of *p*-aminobenzoic acid with glutamic acid, not for those with other amino acids.

Lutwak-Mann (195) observed an inhibiting effect of salicylates

on dismutation reactions and carboxylatic fission in individual en-
zyme systems. The fermentation of glucose is affected by cinchophen.
The inhibition of enzyme systems of yeast is treated in an investigation
of von Euler & Ahlström (196). The influence of salicylic acid,
salicylic aldehyde, acetyl salicylic acid, nicotinyl salicyl amide, etc.,
on growth and different components of the zymase system is more or
less marked according to the concentration chosen.

Stokes (197) reported that pyocyanine, hemipyocyanine, and
thyrothricin have a definite fungistatic effect on yeasts. According to
Segal (198) the inhibiting effect of higher alcohols on the alcoholic
degradation of sugar increases with molecular weight in homologous
series. In the same way the proliferation of the yeast cells is inhibited
by the alcohols. The disappearance of the cell vacuoles, especially in
young cells, is an indication of the morphological changes; degenera-
tive changes are reversed again by subsequent culture in normal
mediums. To suppress the development of yeasts Neiding & Burrell
(199) prefer to use the propylester of p-hydroxybenzoic acid rather
than the methyl ester.

Von Euler & Skarzynski (200) have studied the effect of ultra-
sonic waves on yeast. They observed an effect on the cell structure;
dried yeast and yeast enzymes are hardly altered. Although the cells
are not disrupted, their tendency to grow diminishes and stops in pro-
portion to the length of exposure. According to von Euler, Ahlström
& Högberg (201) the effect of x-rays on fermentation and respiration
of the irradiated cells is small. Similarly, the nucleic acid content and
the rate of growth are changed little.

Hofstetter, Leichter & Nord (202) studied the course of the fer-
mentation with living yeast and maceration juice from a thermo-
chemical point of view. The authors state that the phosphorylation of
sugar in cell-free fermentations is accompanied by the liberation of
20 to 22 calories per molecule of esterified phosphate. This is the
order of magnitude found for the heat of reaction in the normal fer-
mentation of sugar. Data about the temperature coefficient of alcoholic
fermentation are given by Rahn (203).

Phosphatases.—In order to avoid repetition, reference may be
made to recent reviews, treating the part played by phosphatases (and
phosphorylases) in the synthesis, degradation, and conversion of con-
stituents of yeast or in the utilization of offered substrates (86 to 94,
204 to 207). The following publications may be mentioned. The
ability to separate top yeast phosphatase differs according to the yeast

species. Schäffner & Krumey (208) obtained best results after previous drying. Kobayashi (209) claims that extraction of dried yeast with an acetate-cyanide buffer yields a homogenous enzyme. Its specificity is not strongly marked; the enzyme acts on α- and β-glycerophosphate, fructose diphosphate, phosphoglyceric acid, adenylic acid, diphenyl pyrophosphate, and adenosinetriphosphate as well as on other substrates. The optimum pH is 4; above a pH of 7 inactivation can be observed. Noteworthy is the fact that a suspension of yeast, in which Neuberg & Karczag (210) have discovered the phosphatase effect of yeasts, acts exactly like the liberated enzyme. Messart & Dufait (211) and also Messart & Vandendriessche (212) further report the existence of a yeast phosphatase which splits β-glycerophosphate at an optimum pH of 6 to 7. A significant result for the theory of phosphatase effects is that of Schmidt & Thannhauser (213), who found that highly purified alkaline phosphatase from intestines splits both the ester linkages of diphenyl phosphate.

Eastcott & Rae (214) studied the influence of the culture medium on the phosphatase content of yeast. Yeast grown in badly buffered solutions is poor in phosphatase; yeast grown in tomato juice is rich in phosphatase. The phosphate content of the culture medium may be kept low; variations in the magnesium concentration do not affect the result.

Westenbrink & van Dorp (215) showed that unlike animal phosphatase, top yeast phosphatase is strongly inhibited by thiamine. The hydrolysis of both α- and β-glycerophosphate and of thiamine pyrophosphate is decreased substantially in the presence of thiamine. Westenbrink & Veldman (216) found in yeast an enzyme which stimulates hydrolysis and synthesis of thiamine pyrophosphate. The synthesized product is not present in the apocarboxylase fraction.

A publication by Kalckar (217) gives an excellent summary about the function of phosphates in enzymatic synthesis. It deals with the energy relations in a very informative way and also gives the relevant data.

The ability of yeast to split esters of pyrophosphoric acid and inorganic pyrophosphate has been known for a long time. Neuberg & Fischer (218) and Bauer (219) proved the specificity of pyrophosphatase by demonstrating that triphosphoric acid, a polyphosphate more highly condensed than pyrophosphate, is decomposed by another enzyme. Bailey & Webb (220) have worked on the purification of this enzyme. They isolated pyrophosphatase in the following way:

bakers' yeast was autolyzed, the raw enzyme solution dialyzed, and
the enzyme fractionally adsorbed on calcium phosphate gel, washed
out with 10 per cent ammonium sulfate, fractionally precipitated with
solid ammonium sulfate, and freed from the ammonium sulfate by
dialysis. Enrichment is about 130 fold; optimum pH is 6.9 to 7.
The enzyme is active only in the presence of magnesium, which can-
not be substituted by calcium, manganese, zinc, or beryllium. It is
supposed to be a sulfhydryl enzyme. This pyrophosphate is strongly
specific for its inorganic substrate. It is remarkable that the enzyme
is completely inactive when the substrate concentration is greater
than $0.03 M$ and that calcium in concentrations half that of mag-
nesium also completely inactivates the enzyme. This behavior is ana-
logous to that of the common non-specific top yeast phosphatase. Ac-
cording to Albers (221) it too is inhibited more and more by increas-
ing amounts of magnesium.

In the course of investigations on pyrophosphatase at the Dahlem
Institute, Kitasato (222) discovered metaphosphatase, which hydrates
the salts of metaphosphoric acid $[(MPO_3)_3$ or $(MPO_3)_6]$ to ortho-
phosphates. (If the formula of the metaphosphate is written as

$$
\begin{array}{c}
\text{OH} \\
\text{O} - \text{P} - \text{O} \\
\text{HO} \cdot \text{P} : \text{O} \quad \text{O} \quad \text{O} : \text{P} \cdot \text{OH,} \\
\text{O}
\end{array}
$$
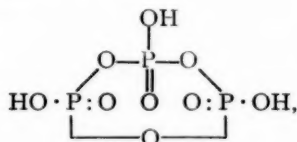
the transformation looks like a hydrolysis instead of a hydration.)
While Lohmann (223) failed to find the enzyme, Neuberg & Fischer
(224) conclusively established its existence. They demonstrated that
the fission of an equimolecular mixture of sodium metaphosphate and
sodium pyrophosphate by yeast enzyme differs completely from the
hydrolysis of sodium triphosphate which, according to the formula
$Na_5P_3O_{10}$, has the same composition as the mixture $NaPO_3 +
Na_4P_2O_7$. The existence of the metaphosphatase has been confirmed
by Schäffner & Krumey (225) as well as by Bamann & Heumüller
(226). Its optimum pH is about 7. The enzyme is not identical with
yeast pyrophosphatase, since it is destroyed after heating at 50° for
ten minutes, while pyrophosphatase remains stable. [A strong meta-
phosphatase has recently been found by Mann (227) in *Aspergillus
niger*. At the same time Mann also confirmed Hardin's almost for-

gotten report (228) that metaphosphates occur in nature together with pyrophosphates.]

In 1937 Neuberg & Fischer discovered triphosphatase which splits the pure inorganic triphosphoric acid

$$(HO)_2P-O-\overset{\displaystyle OH}{\underset{\displaystyle O}{\overset{\displaystyle \|}{P}}}-O-P(OH)_2$$
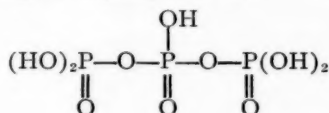
into orthophosphate. The authors (218, 224, 229) proved the specific character of the enzyme by demonstrating that simple plasmolysis of fresh bottom yeast yields a solution which has no effect on triphosphate, but hydrolyzes pyrophosphate and different phosphoric acid esters. The triphosphatase can be detected in intact bottom yeast, however, and after previous drying of the yeast is set free by a special method of autolysis. When top yeasts are treated in the same way soluble triphosphatase is obtained. The enzyme rapidly splits the substrate in neutral or slightly alkaline reaction, especially in the presence of magnesium. Needham *et al.* (230) confirmed the specific character of the enzyme. Schäffner & Krumey (225) and also Bamann & Heumüller (226) put forward a further argument in favor of the specificity of the enzyme, i.e., that the triphosphatase is destroyed by heating at 50° exactly like the metaphosphatase (see page 000), while the pyrophosphatase remains active. The poorly specific top yeast phosphatase also hydrolyzes sodium triphosphate at pH 4. Apparently a second triphosphatase exists, which is inactive on this substrate at a pH equal to or above 7. According to reports by Neuberg, Frankenthal & Roberts (231) American yeasts, which differ in some respects, also contain triphosphatase, top yeasts about 20 per cent less than bottom yeasts. The enzyme is present in the maceration juice of both yeast species. It is possible that in addition to other functions the triphosphatase also has the character of a protective enzyme (similar to the catalase). Adenosinetriphosphate is a derivative of triphosphoric acid found in nature. The purely inorganic compound has certain properties which might necessitate its removal during metabolism, e.g., the property of forming soluble complexes with calcium fluoride, tricalcium phosphate, barium sulfate, magnesium ammonium phosphate, and calcium carbonate, as well as with iron, copper, zinc, and manganese.

The carboligatic reaction [see Neuberg & Ohle (232)] and its

relation to metabolic processes has increased in significance during recent years. The condensation to ketols similar to acetoin which according to the formula appears to be the linking of two aldehydes, was discovered twenty-five years ago by the formation of phenyl acetyl carbinol when fermenting yeast came in contact with benzaldehyde. This condensation differs completely from the aldol condensation. It can be brought about without any difficulty if the acetaldehyde which furnishes the acetyl group is in *statu nascendi* during the fermentation of sugar or pyruvic acid. During this linking of two aldehydes, which so far could not be accomplished by purely chemical means, the compound $C_6H_5 \cdot CHOH \cdot CO \cdot CH_3$ (I) is formed from benzaldehyde. Years ago Neuberg & Ohle (232) proved that (I) and not the isomeric methyl benzoyl carbinol $C_6H_5 \cdot CO \cdot CHOH \cdot CH_3$ (II) is formed. Von Auwers (233) who previously thought that the biochemically produced ketols must have the constitution (II) has given up his opinion and found that the formulas which he gave to the two isomers for many years were inadequate and must be exchanged. Agreement has thus been reached on the constitution of the carboligatic product. The fact that ephedrin, $C_6H_5 \cdot CHOH \cdot C(NHCH_3) \cdot CH_3$, is made from the biochemical product of yeast and benzaldehyde by a simple process which is extensively used in industry today also shows, quite independently, that the formula of Neuberg & Ohle is the correct one. A further proof for the constitution I has been given recently by Berg & Westerfeld (234). Since the simplest ketol, acetyl methyl carbinol or acetoin is a practically omnicellular product, the importance of the carboligatic reaction is obvious.

The question whether its formation is due to a special enzyme (carboligase) or whether the ketol condensation is a secondary effect of the carboxylase as presumed by Dirscherl (235) has been clarified by Hofmann (236) in the sense that the reaction is a special enzymatic one. The optical activity of all acetoins further favors this view. The formation of an optically active ketol from two structurally inactive aldehydes can only be explained by making certain assumptions. When considering the addition of 1 mol aldehyde to pyruvic acid, e.g., according to the formula

$$CH_3 \cdot C(OH) \cdot COOH$$
$$| \atop OC \cdot R$$
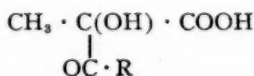
as already thought of when the carboligatic reaction was discovered, it can be observed that an asymmetrical carbon atom is produced. But such an association of the components will not take place freely. Unpublished experiments show that 1-valeraldehyde and pyruvic acid on the one hand, and acetaldehyde and 1-methyl ethyl pyruvic acid (237) on the other hand are miscible without change of rotation and properties at 18 to 42°C. The participation of a biocatalyst must thus be assumed. If this were the carboxylase by itself we would be dealing with a second function of the same enzyme, while a third function would become manifest if it decarboxylated this β-ketonic acid as well. Transformation of the primarily produced ketol to the isomer with the second constitution would be a fourth function, during which the optical activity would have to be preserved. It seems unwarranted to ascribe these different functions to the highly specific ordinary α-carboxylase. Silverman & Werkman (238) working with an enzyme from *Aerobacter aerogenes* also arrived at the conclusion that the enzyme carboxylase could not be responsible for the formation of acetyl methyl carbinol from pyruvic acid. While the decarboxylation of pyruvic acid by yeast does not require inorganic phosphate the carboligase of Silverman & Werkman needs inorganic phosphate; the formation of a phosphorylated intermediate is probable. With the new enzyme the transformation of pyruvate into acetoin is quantitative; acetaldehyde, the typical product of the true carboxylatic fission, does not occur at all. The specific rotation of the acetyl methyl carbinol formed has been found to be $[\alpha]5460 = -100°$.

Apart from acetaldehyde and benzaldehyde Neuberg & Liebermann (239) as well as Behrens & Ivanov (240) previously showed the ability of *o*-chlorobenzaldehyde, *o*- and *p*-toluylaldehyde anisaldehyde to enter into ketol formation. A further aliphatic example is propionic aldehyde, which is transformed to propionyl methyl carbinol. This has been proved by Green *et al.* (241) as well as by Berg & Westerfeld (242). The same authors (243) proved a ketol condensation of individual acetaldehyde, i.e., which did not have to react in *statu nascendi,* with an enzyme from heart muscle. A carboligatic synthesis thus probably exists independently from the effect of the carboxylase. Tomiyasu (244, 245, 246) also came to the same conclusion. He too demonstrated the high specific rotation of acetoin ($[\alpha]_D$ up to $-98.3°$). A higher value has also been determined for the rotation of 2,3-butylene glycol photochemically produced by means

of yeast. Stotz, Westerfeld & Berg (247) found that the practically quantitative formation of acetoin from acetaldehyde and sugar or pyruvic acid and aldehyde with yeast takes the same course with homogenized brain (brain brei).

An unexpected function of carboligatic products in metabolism has been pointed out by Doisy & Westerfeld (248). Acetyl methyl carbinol, like 2,3-butylene glycol which is closely related to it, can take part in the processes of biological acetylations.

Antoniani & Gugnomi (249) showed that the acetoin is preserved during the fermentation of wine with pure cultures of *Pseudosaccharomyces apiculatus* and *P. magnus,* while a phytochemical reduction to 2,3-butylene glycol predominates in the fermentation with *S. ellipsoideus.* According to Antoniani & Castelli (250) this reduction is the rule with mixed cultures under natural conditions. A further communication (251, 252) gives information about the time factors involved. The formation of products of carboligatic synthesis cannot be observed when grape juice is fermented with *Hansenula panis* under normal conditions, but is found after the addition of calcium carbonate or ascorbic acid. Antoniani (253) proposes a classification of pure yeasts according to their ability or inability to accumulate acetoin under normal conditions.

Kamlet (254) describes the preparation of 1-ephedrine, starting with 1-acetyl phenyl carbinol, the product of the carboligatic synthesis during the fermentation of sugar in the presence of benzaldehyde.

Improved (255) and refined (256) methods for the determination of carboligatic products have been described by Tomiyasu, Stotz & Reberg, as well as by Berg & Westerfeld (257). Quantities of 1 ml. and less may be determined by the method of Neuberg & Strauss (258).

REDUCTIONS

Due to the easy accessibility of yeasts it is logical that numerous morphological and genetical investigations have been made with them. Only a few publications in this field can be mentioned here.

Nilsson (259) illustrates in an extensive summary the influence of the cell structure on the course of fermentation phenomena. In particular the questions of localization of the enzyme, of morphological organization, and of the behavior of structureless systems are discussed. Nilsson favors the view that lipid-proteins are playing an essential part as carriers of enzymes and therefore should be present

in enzyme preparations in order to reproduce conditions in intact cells in such systems. Mention should also be made of the ideas put forward by Cohen (260) on a possible experimental approach to the organization of protoplasm.

The discovery of new strains of yeast is of interest for special questions of fermentation as well as for industry. Giovannozzi (261) isolated four asporogenous yeasts and different sporogenous strains of a new species of the so-called debaryomyces type from fermenting Kentucky tobacco. Leshinskaya (262) isolated a new yeast strain of high fermenting activity. It seems to be a top yeast, since it is compared with strain XII (German yeast). It has been named SH. Hongo (263) cultivated five varieties of lactose fermenting yeast, four of which are new, from fermentation products of milk. They belong to the Torula respectively the *Torulopsis* group. Baba (264) communicates the discovery of dextrin-fermenting yeasts, isolated from cassava starch. The products of fermentation are the usual ones.

Fosdick & Starke (265) state that there exists a symbiotic relationship between yeast and *B. acidophilus*. This is an organism occurring in the mouth. Various types of carbohydrate transformations are catalyzed by these micro-organisms living in symbiosis.

Among Nickerson's papers his publication with Thimann (266) may be singled out for special attention. The authors discovered a substance promoting conjugation. This substance, which is soluble in 90 per cent alcohol, is liberated when yeast cells die off, but separates in larger amounts in cultures of *Aspergillus niger*. It is not specific, since the normally rare conjugation of three different strains of zygosaccharomyces could regularly be achieved with it, too. According to Cook *et al.* (267, 268, 269) factors promoting proliferation are found in the cell-free filtrate of *S. cerevesiae* cells which have been damaged by treatment with ultraviolet light. In addition to known growth substances the filtrate contains an unknown factor. Nucleosides and adenylic acid have a part in this effect. Nyberg (270) isolated from a spore-forming, S, strain of top yeast an R form, which no longer was spore forming. After uniting of the R cells, R and S forms arise. Continuing the culture of S the development of a mutant S' could be observed to a small extent.

According to Rhoades (271) the variation in the fermentation abilities may have two causes. The mutant may occur or an adaptive enzyme may be formed by a special chemical stimulation of the cell. The enzymes are divided into constitutive enzymes, always formed

independently of the composition of the offered nutriment, and adaptive enzymes, produced only when the cells are grown in the presence of the specific substrate. The ability to form adaptive enzymes is inherited by the cell. Once formed, this ability can be lost by passage of the cell through different substrates. Glucose, mannose, saccharose, and raffinose are fermented by every strain, regardless of the source of carbon in the nutrient solution. If the solution contains galactose, maltose, α-methyl glucoside, trehalose, or melezitose, adaptive enzymes are developed. Galactozymase is constitutive for *S. cerevisiae* only. In other strains which belong to the groups *S. ellipsoideus, carlsbergensis,* and *schizosaccharomyces pombe,* galactose is fermented only by the adaptive enzyme. α-Methyl glucosidase is constitutive in *S. pombe,* adaptive in *S. ellipsoideus.* Maltase is formed when the yeast is grown in α-methyl glucoside, maltose, or melezitose, but occurs in some yeasts as a constitutive enzyme. (The fact that α-methyl glucoside causes the development of typical maltase is of interest for the question of direct fermentation of maltose, since the proofs for this are partly based on the different behavior of maltose and α-methyl glucoside as substrate.) Kosterlitz (130, 272) assumes that during the fermentation of galactose two adaptive enzymes take part: first, one that produces galactose-1-phosphate, and then a second one which brings about transformation to the glucose series, possibly by way of glucose-1-phosphate. From this fructose diphosphate, already obtained by Nilsson from galactose in 1930, is then formed in the usual way. Whether the step of triose phosphate is involved in this transformation has yet to be solved.

It can be seen from a study by Lindegren & Spiegelmann (273) that more than 1,450 genetically different strains of *S. cerevisiae* are known. The significance of this fact alone clearly shows that we are only at the beginning of our knowledge about the comparative biochemical behavior of different types of yeast. The relation of sporulation and the range of variation to adaptation, Mendelian and cytoplasmic inheritance in yeasts, the possibility of breeding yeasts of special industrial value by selection and hybridization all form the basis of entirely new perceptions and problems in yeast research. Within the limits of this review mention can only be made of the newest results of Winge (274) and the school of Lindegren (275). Many contradictory reports about the fermentability of different sugars by different yeasts find an unexpected illustration here. It is also possible that the problems of direct fermentation of di- and polysac-

charides, i.e., without previous hydrolysis to monosaccharides, require renewed and intensified study. A summary by Leibowitz & Hestrin (276) gives information about the present state of this problem. Recently Doudoroff (277) contributed to this question by experiments, which were not made with yeast but with *Pseudomonas saccharophila*. He found active intracellular melibiase, invertase, and phosphorylase in dry cells but not in the culture medium. The monosaccharides are attacked with greater difficulty than complex sugars.

To judge the actual position, ideas put forward by Kluyver & Custers (278) deserve thorough study. The authors claim that during so-called direct fermentation of disaccharides intermediate compounds with a number of carbon atoms between 7 and 11 and still containing the oxygen bridge of the starting material will occur. No experimental evidence for such a process has been brought forward so far ; the authors show, however, that all yeasts which assimilate and oxidize disaccharides contain in greater or lesser quantity the corresponding hydrolases. Under anaerobic conditions these hydrolases are inactivated totally or in part. According to Kluyver & Custers this phenomenon[1] can be explained in different ways. It seems quite possible that the increased state of proteolysis which is characteristic for the cell under anaerobic conditions is the main cause of the intracellular inactivation of the carbohydrases which are proteins or bound to proteins.

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BACTERIAL METABOLISM

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This review attempts to cover the literature of the past three years (1943 to 1945) dealing with oxidative and fermentative metabolism, certain assimilatory processes such as carbon dioxide utilization, and the enzymes taking part therein. Due to lack of space a number of important subjects including bacterial nutrition, antibiotic substances, the action of chemotherapeutic agents and the chemistry of cellular constituents, have been completely omitted. It is hoped that these subjects will be covered in other reviews.

Chemo-Autotrophic Metabolism

It has always been difficult to understand how *Thiobacillus thiooxidans* and other bacteria can utilize elementary sulfur in view of its insolubility in water. Recently[1] evidence was presented indicating that this is accomplished by a preliminary dissolution of the sulfur in globules of highly unsaturated fat located in the ends of the cells. Although this explanation may be correct in principle, it is not consistent with all the facts now available. Recent cytological observations (1) show that there can hardly be any direct contact between the fat-like globules within the cell and the sulfur in the external medium since they are separated by a protoplasmic layer, the cell wall, and a rather thick capsule. A complete explanation for the entry of elementary sulfur into the cell remains to be found.

Thiobacillus can survive for long periods in the absence of its specific substrate, sulfur. Under such conditions reserve organic materials, variously identified as polysaccharide and volutin, are oxidized to carbon dioxide. Analysis of the organic phosphorus compounds in cell extracts indicates that the organic metabolism of *Thiobacillus* is essentially like that of other living organisms (2). Adenosinetriphosphate, fructose-1,6-diphosphate, phosphoglyceric acid, fructose-6-phosphate, glucose-6-phosphate, glucose-1-phosphate, and coenzyme I have been identified with a fair degree of certainty. Variations in the proportions of these compounds with the nutritional state of the organism definitely indicate they are involved in active metabolism. The adenosinetriphosphate of *Thiobacillus* differs from that of

[1] *Ann. Rev. Biochem.*, 12, 564 (1943).

other organisms so far studied in having the phosphate attached to the third rather than the fifth carbon atom of ribose (3).

An investigation of hydrogen utilization by *Hydrogenomonas flava* by manometric methods has shown that the hydrogen utilizing system is adaptive (4). Autotrophically grown cells can oxidize both hydrogen and lactate, either separately or together. The oxidation of lactate does not decrease the rate of hydrogen oxidation; in fact, the latter is generally stimulated. The old theory of Kaserer that hydrogen utilization by bacteria of this type involves a direct reduction of carbon dioxide to carbohydrate is refuted by the demonstration that no reaction between hydrogen and carbon dioxide occurs anaerobically. Even more conclusive evidence on the same point was obtained in unpublished experiments by Doudoroff & Ruben by the use of C^{11}. No significant utilization of labeled carbon dioxide occurred anaerobically in a hydrogen atmosphere. But when oxygen was present, carbon dioxide was taken up even when endogenous respiration was the only source of energy. The quantity of carbon dioxide used by bacteria supplied with hydrogen and oxygen is relatively independent of the oxygen-carbon dioxide ratio in the atmosphere, but differs considerably with the species and strain (5). From this it appears that the energy utilization mechanisms are not identical in all hydrogen bacteria.

Various biochemical problems of the chemo-autotrophic bacteria have been discussed by van Niel (6).

CARBON DIOXIDE UTILIZATION BY HETEROTROPHIC BACTERIA

Until last year (1944) the only well-supported mechanism for carbon dioxide fixation involving formation of a carbon to carbon bond was the C_1—C_3 addition reaction, formulated as follows:

$$CO_2 + CH_3COCOOH = COOHCH_2COCOOH.$$
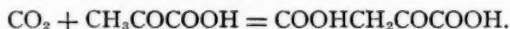
This fixation reaction can readily account for the synthesis of succinic and other C_4-dicarboxylic acids from carbon dioxide. It is also adequate to explain the synthesis of pyruvate, lactate, and propionate when there is independent evidence for the intermediate formation of dicarboxylic acids. But when the latter are not formed and apparently not metabolized, as in certain butyric acid fermentations, an explanation of the synthesis of C_3 compounds which involves the C_1—C_3 addition reaction can be accepted only with strong reservations.

Recently a second mechanism for the entry of carbon dioxide into the carboxyl group of C_3 compounds was discovered (7, 8) which

involves two steps: (*a*) a reduction of carbon dioxide to formate, and (*b*) a reaction between formate and acetyl phosphate to give pyruvate and inorganic phosphate:

$$CH_3COOPO_3H_2 + HCOOH \rightleftharpoons CH_3COCOOH + H_3PO_4.$$
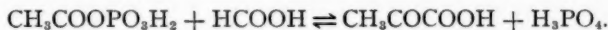
Both steps are reversible.

A third possible fixation mechanism is very similar to the second but does not involve formate:

$$CH_3COOPO_3H_2 + CO + H_2 \rightleftharpoons CH_3COCOOH + H_3PO_4.$$
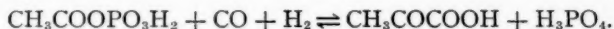
The forward reaction has not been demonstrated directly but various types of indirect evidence indicate that it occurs under the influence of an enzyme present in *Cl. butylicum* (7, 9). The decomposition of pyruvate by both of the above reactions is discussed in more detail in another section of this review.

The only simple compounds whose synthesis from labeled carbon dioxide (C^*O_2) cannot be explained by the above reactions are methane, and acetic acid containing two labeled carbon atoms (see below). Carboxyl-labeled acetic acid may be formed by splitting of carboxyl-labeled succinic acid (10).

Most heterotrophic bacteria that use carbon dioxide incorporate it only into the carboxyl groups of organic acids like succinic, lactic, or acetic acids or their derivatives. Until recently only two heterotrophic organisms, *Cl. aceticum* and *Cl. acidi-urici*, were known to use carbon dioxide for methyl as well as carboxyl group synthesis; both species make acetic acid from carbon dioxide. Now two more anaerobic bacteria, *Cl. thermoaceticum* and *Butyribacterium rettgeri*, have been shown to carry out the same type of reaction. *Cl. thermoaceticum* ferments (11, 12) glucose and xylose with the formation of acetic acid as the only product and ferments pyruvate according to the equation:

$$4 CH_3COCOOH + 2 H_2O = 5 CH_3COOH + 2 CO_2.$$
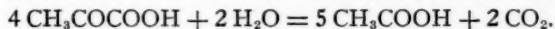
The remarkable features of all three fermentations are the high yield of acetic acid and the low yield or absence of carbon dioxide. Experiments with C^{14} show that in the glucose fermentation approximately two moles of carbon dioxide are formed per mole of substrate decomposed but they are immediately reused for acetic acid synthesis. The data are therefore in accordance with the following equations:

(*a*) $C_6H_{12}O_6 + 2 H_2O = 2 CH_3COOH + 2 CO_2 + 8 H$

(*b*) $8 H + 2 CO_2 = CH_3COOH + 2 H_2O.$
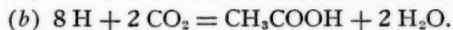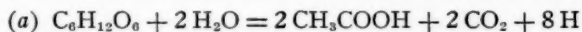
Somewhat similar results have been obtained with *B. rettgeri* (13, 14). This organism ferments lactate to acetic and butyric acids and carbon dioxide. Experiments with C^{14} showed that at least 0.8 mole of carbon dioxide is produced per mole of lactate and about half of this is then converted into acetic and butyric acids. All the carbon atoms of these acids contain carbon dioxide carbon more or less equally distributed. Acetic acid is shown to be the primary product and is subsequently partially converted to butyric acid.

In a study of the kinetics of methane formation from carbon dioxide and gaseous hydrogen catalyzed by *Methanobacterium omelianskii* (15) it is shown that the rate is greatly dependent upon carbon dioxide concentration. The Michaelis constant for the reaction is 7×10^{-5} M. This is higher than the value for photosynthesis by algae (1 to 7×10^{-6} M) and considerably lower than that for the formic hydrogenlyase reaction of *E. coli* (1.5×10^{-3} M).

Cl. butylicum has been added to the already long list of bacteria that fix carbon dioxide in the carboxyl groups of organic acids (16). In this instance the carbon dioxide carbon is found in lactic, pyruvic, and formic acids, but not in acetic and butyric acids or the corresponding alcohols. The results cannot be explained by a $C_3 - C_1$ addition reaction since dicarboxylic acids are not metabolized at an adequate rate. The authors are inclined to favor a mechanism involving a $C_2 - C_1$ addition reaction such as that between acetylphosphate and either formate or carbon dioxide and hydrogen.

Since a great deal of indirect evidence indicates that the carboxylation of pyruvic acid to give oxaloacetic acid is a fundamental reaction in carbon dioxide fixation, efforts have been continued to demonstrate this reaction directly. A β-decarboxylase catalyzing the conversion of oxaloacetate to pyruvate and carbon dioxide was found in *Micrococcus lysodeikticus* in 1941 (17), but the reversibility of the reaction could not be demonstrated. However, it has now been found that during the enzymatic decarboxylation of oxaloacetate in the presence of C^*O_2 some of the isotope gets into the carboxyl group adjacent to the methylene group of the C_4 acid (18). A greater exchange of labeled carbon occurs with the product of the enzymatic oxidation of fumarate than with synthetic oxaloacetate. This suggests that the two substances are not identical. With a cell-free enzyme preparation from *E. coli*, the formation from pyruvate and carbon dioxide of a compound having properties similar to oxaloacetate is reported (19). Although the quantity of the compound is small, it nevertheless appears to be more

than could be expected on the basis of the ΔF of the carboxylation reaction.

The replacement of the carbon dioxide requirement of the meningococcus by a mixture of guanine, uracil, and cytosine is of great interest (20). Vitamin-free casein hydrolyzate is also partially effective.

OXIDATIVE METABOLISM

On the basis of rates of oxygen uptake by cultures of several acid-fast and selected nonacid-fast bacteria with a large number of substrates (21), the not unexpected conclusion was reached that related species generally show a similar type of metabolism. The grouping of bacteria into lipophiles, glycophiles, and proteophiles on the basis of apparent preference for fatty acids, sugars, or amino acids seems hardly justifiable in view of the small number of organisms studied and the fact that they were not grown in the presence of the particular substrates on which they were tested. The respiratory activity of the acid-fast bacteria, classed as lipophiles, was found to decrease generally with increasing acid-fastness. It has been shown in somewhat similar experiments that different species of *Mycobacterium* vary widely with respect to the rates of oxygen consumption in the presence of different substrates (22).

Although the oxidation of acetate by *Bacterium turcosum* (23) proceeds at several times the rate of succinate oxidation, the possibility that the latter compound may be an intermediate in acetate utilization is not excluded, since the permeability of the cell membrane may be the limiting factor in succinate oxidation. One third to one half of the acetate is not completely oxidized, but is probably assimilated. Assimilation is reduced or suppressed in cells from old cultures as well as in those poisoned with iodoacetate.

Certain streptococci are capable of oxidizing butyrate with the production of hydrogen peroxide (24). Under the conditions of the experiment, methylene blue could not be shown to serve as hydrogen acceptor for this oxidation.

The oxidation of succinic, malic, and fumaric acids by resting cells of *Pseudomonas saccharophila* results in the assimilation of approximately one half of the substrate carbon (25). Under suitable conditions, the oxidation of all three substrates proceeds in two stages, distinguished by different rates of oxygen uptake. The first stage may be interpreted as an oxidation of the substrate to pyruvate, which was

shown to accumulate, while the second stage involves the oxidation and assimilation of pyruvate. Young cells and cells grown in iron-deficient media do not show two distinct rates. Oxaloacetate is not decomposed anaerobically and its oxidation does not result in either pyruvate accumulation or the same amount of assimilation as observed with the more reduced substrates.

An interesting and as yet unexplained effect of a number of amino acids on the rate of pyruvate oxidation was observed in studies with a pneumococcus (26). The rate of utilization of pyruvate, but not of glucose, is increased 10 to 130 per cent by the addition of such compounds as phenylalanine, leucine, isoleucine, valine, serine, unheated serum albumin, and haemoglobin. Alanine and lysine have little if any effect, while glutamic acid is active only in acid media. The d- and l-forms have approximately equal activities, and neither deamination nor other decomposition of the amino acids appears to be involved in their catalytic action. The oxidation of pyruvate by $E.$ $coli$ can be greatly accelerated by the addition of aspartate or glutamate, and, to a lesser extent, of ammonia (27). The effect of the amino acids is interpreted as being due to the combined catalytic action of ammonia and of the dicarboxylic acids liberated on deamination. It is not clear how growth of the organism was avoided in the rather prolonged manometric experiments with a suitable nitrogen source present, and a conclusive demonstration of the fate of the added amino acids is lacking in these experiments.

A detailed account of the experimental work on the oxidation of pyruvate by $Bac.$ $acidificans$ $longissimus$ $(L.$ $delbrueckii)$ has recently been published [(28), see also (28a)]. Acetylphosphate has been positively identified as an oxidation product by isolation and analysis of the pure silver salt. Acetylphosphate enters into a reversible reaction with adenosinediphosphate to form acetic acid and adenosinetriphosphate. From the approximate equilibrium constant of the reaction it is calculated that the acyl phosphate bond energy is about 15 Kcal. Acetylphosphate is now recognized to be an intermediate in anaerobic as well as aerobic pyruvate decomposition by bacteria (9, 29).

A different mechanism of aerobic pyruvate oxidation to acetic acid and carbon dioxide appears to be involved in the metabolism of $Proteus$ $vulgaris$. The pyruvic oxidase obtained from this organism (30) appears not to require phosphate for its activity and acetylphosphate could not be demonstrated as a product of the reaction.

Neisseria intracellularis, unlike *N. gonorrhoeae,* can grow aerobically in a simple medium containing glutamate and glucose or lactate as the only organic compounds (31). The catabolism of the organism apparently consists of the oxidation of the glucose or lactate via pyruvate to acetate and carbon dioxide. The reactions of glutamate are unfortunately not considered.

Alcohol can be oxidized to acetic acid by *Streptococcus mastiditis* without the accumulation of hydrogen peroxide (32). With aldehyde fixative, a rapid oxidation of alcohol proceeds with the uptake of 0.5 mole of oxygen per mole of alcohol. Aldehyde can be further oxidized to acetic acid, or dismutated to alcohol and acetic acid under anaerobic conditions. Since alcohol oxidation is inhibited by 0.01 M iodoacetate, while both the oxidation and the dismutation of aldehyde are insensitive, the formation of acetic acid from acetaldehyde must be directly linked to oxygen utilization rather than depending on the dismutation of the aldehyde and the aerobic oxidation of alcohol.

Hydrogen peroxide is formed in the aerobic oxidation of glycerol to lactic acid by *Streptococcus faecalis* (33). Glycerophosphate and triosephosphate appear to be intermediates in this oxidation.

Acetobacter suboxidans can oxidize the meso, but not the $l(+)$ form of 2,3-butanediol to acetoin (34). Likewise, *Aeromonas hydrophila* forms acetoin from the meso, but not from the $l(+)$ or $d(-)$ isomers, although it produces both $d(-)$ and meso-butanediol in fermentation. Both the meso and $d(-)$, but not the $l(+)$, isomers are oxidized to acetoin by *Aerobacillus polymyxa.* On the other hand, *Aerobacter aerogenes* uses only the meso and $l(+)$ forms, both of which it produces fermentatively. The acetoin can be further oxidized by this organism, with the assimilation of approximately half of the substrate carbon to cell material. Both *A. aerogenes* and *A. polymyxa* can oxidize 1,2-propanediol to acetol but are unable to attack 1,2-ethanediol, 1,3-butanediol, or 2-methyl-1,2-propanediol (35).

The oxidative utilization of glucose during the growth of *Pasteurella pestis* results in the accumulation of rather large quantities of pyruvic acid, which later disappear from the medium (36).

The extent of oxidative assimilation of glucose as well as other substrates by resting cells of a number of bacterial species may be roughly estimated from manometric data on oxygen consumption (37). In suitable concentrations, azide and dinitrophenol increase the amount of oxygen uptake accompanying the utilization of glucose. This increase is presumably due to the partial or complete blocking of anabolic

reactions. However, the danger of relying on manometric data alone as a criterion of assimilation has been clearly shown in experiments with yeast (38). With *Achromobacter fischeri* dinitrophenol appears to decrease rather than increase the fraction of glucose appearing as carbon dioxide in respiration (39). The effect of chloral hydrate and chloretone on the rate of glucose oxidation by this organism can best be interpreted as being due to an increased rate of breakdown of cellular constituents. Chloretone stimulates the endogenous respiration which follows the exhaustion of glucose metabolized in the presence of this poison. When, on the other hand, glucose is oxidized in the presence of chloral hydrate, the subsequent autorespiration is not stimulated by chloral hydrate, unless the cells are temporarily removed from the poison. These observations, as well as the behavior of dinitrophenol-poisoned cells with regard to respiration and luminescence, suggest that the various toxic agents have specific effects in determining the nature of anabolic products.

In studies with *Pseudomonas saccharophila* (5), a fair correlation could be shown between assimilation during growth and assimilation by resting cells when glucose was used as substrate. With lactic, pyruvic, and acetic acids, progressively greater discrepancies appear between long growth experiments and short manometric experiments. Fairly wide variations in temperature, composition of the atmosphere, and pH of the medium have little effect on the fraction of glucose assimilated by growing cultures. Removal of carbon dioxide, high oxygen tensions, and insufficient iron decrease assimilation. With limiting iron concentrations, growing cultures produce pyruvic acid from sucrose and trehalose, but not from glucose. The demonstration of the phosphorolytic breakdown of sucrose by this organism does not explain either the high rate of utilization of the fructose component of this disaccharide as compared with that of fructose supplied in the medium or the observed high rates of oxidation of trehalose and melibiose (40, 41, 42). An important and often disregarded effect of substrate concentration on the utilization rate was observed in these studies. In low concentrations, sucrose and raffinose are utilized at a much greater rate than melibiose, while at high concentrations, the rate of melibiose oxidation is highest.

Cholesterol is decomposed aerobically by several species of *Mycobacterium* (43) and *Proactinomyces* (44). With the latter, the accumulation of an intermediate product of incomplete oxidation could be demonstrated. This product, Δ^4-cholestenone, results from the oxi-

dation of the hydroxyl group at C—3. Similar incomplete oxidations of dehydroandrosterone to androstendione and of a number of other steroids of the sex hormone series are effected by *Flavobacterium (Micrococcus) dehydrogenans* (45). The identification of intermediate products in the oxidation of cholic acid to 3,7,12-triketocholanic acid by *Alcaligenes fecalis* (46), has revealed the interesting fact that the hydroxyl groups are oxidized in the same order by bacteria and chromic acid.

Glutamic acid is oxidized by growing cultures of *Ps. fluorescens* with the formation of carbon dioxide, ammonia, and cell material (47). The calculated carbon-nitrogen ratio of the cell material is 2.5, which seems unreasonably low.

Growth factors, like other organic compounds, are decomposed in nature under the influence of bacteria. Riboflavin is readily oxidized by *Pseudomonas riboflavina* to lumichrome and carbon dioxide (48, 49). A theoretical yield of lumichrome is obtained but carbon dioxide production is only about 40 per cent of that required for complete oxidation of the ribityl side chain; the remaining three carbon atoms are presumably used in assimilatory processes. It is not clear whether the ribityl side chain is oxidized directly or whether riboflavin is first hydrolyzed to lumichrome and ribitol followed by oxidation of the latter. A number of intestinal bacteria, both Gram positive and Gram negative types, can decompose ascorbic acid rapidly and completely under anaerobic conditions if an easily fermentable sugar is not simultaneously present in large amount (50). The bacteria appear to use ascorbic acid as a carbon and energy source. *Pseudomonas* and *Serratia* species can either synthesize or destroy nicotinic acid depending on its concentration (51).

Anaerobic Metabolism

Butyric acid fermentations.—Several notable contributions have been made during the last three years to our understanding of butyric acid-butyl alcohol-acetone fermentations. These are (*a*) the discovery of the role of phosphate in the dissimilation of pyruvate, (*b*) the demonstration, by means of carbon isotopes, that acetic acid is converted to higher fatty acids and other products, and (*c*) the demonstration of the role of iron in controlling the course of the *Cl. welchii* fermentation.

The role of phosphate in pyruvate decomposition has been studied with a cell-free preparation of *Cl. butylicum* (9). This was earlier shown to convert pyruvate anaerobically to acetate, carbon dioxide,

and hydrogen. The reaction rate is dependent upon inorganic phosphate concentration and it has been found that during the reaction inorganic phosphate disappears and a labile phosphate compound is formed. The latter could not be isolated, but convincing evidence was obtained to indicate it is a mixture of acetyl- and butyryl-phosphates, the former predominating. The primary reaction can be formulated as follows:

$$\text{pyruvate}^- + H_2PO_4^- \rightleftharpoons CO_2 + H_2 + \text{acetylphosphate}^=.$$

The ΔF of this reaction is evidently very small. The forward reaction can be accelerated either by increasing the inorganic phosphate concentration or by adding glucose which evidently reacts with acetylphosphate via the adenosinetriphosphate system. Hydrogen, on the contrary, inhibits the cleavage of pyruvate (7). The above reaction has not been directly shown to be reversible, but reversibility is indicated by the demonstration that the *Cl. butylicum* enzyme preparation catalyzes a rapid exchange of labeled phosphate between acetyl- and inorganic phosphate.

Butyrylphosphate is evidently formed by a transfer of phosphate from acetylphosphate to butyric acid:

acetylphosphate + butyric acid $\rightleftharpoons$ acetic acid + butyrylphosphate.

This reaction is probably reversible and mediated by the adenosinetriphosphate system (28). Butyrylphosphate may well be the immediate precursor of butyl alcohol in this type of fermentation. From the thermodynamic point of view the reduction of an acylphosphate is much easier than the reduction of a free carboxyl group.

The importance of acetate and possibly acetylphosphate as intermediates in butyric acid fermentations is clearly shown in several studies with carbon isotopes. When starch is fermented by *Cl. acetobutylicum* or *Cl. butylicum* in the presence of carboxyl-labeled acetate, all of the products contain the isotope (52, 53), the highest concentrations being present in butyl alcohol, butyric acid, acetone, isopropyl alcohol, and carbon dioxide. The possibility that carbon dioxide is an intermediate in the formation of the organic compounds could be excluded. Therefore a more or less direct conversion of acetic acid into the above products must be accepted. The formation of acetone and isopropyl alcohol from acetate was to be anticipated since it has long been known that added acetate is almost quantitatively converted to these two products. But the conversion of acetate to butyric acid and

butyl alcohol was unexpected. Acetaldehyde has been generally assumed to be the precursor of the C_4 compounds, though the evidence for this view was quite inadequate. The formation of labeled ethyl alcohol shows that it can be formed by reduction of acetic acid.

The distribution of labeled carbon in butyl alcohol, acetone and isopropyl alcohol formed from carboxyl-labeled acetate provides valuable evidence as to the mechanism of formation of these compounds. In butyl alcohol the labeled carbon is almost equally distributed between the carbinol and beta positions. The alpha and gamma positions are unlabeled. From this it can be concluded that the C_4 chain is synthesized from two molecules of acetic acid or a derivative thereof.

In acetone and isopropyl alcohol the labeled carbon is exclusively in the carbonyl and carbinol groups, respectively. This distribution and the fact that the isotope content of the fermentation carbon dioxide is about the same as that of the C_3 compounds, supports the view that acetone is formed by decarboxylation of acetoacetic acid formed from acetate.

When labeled butyrate was added instead of labeled acetate to a corn mash fermentation, most of the isotope was recovered in butyl alcohol. This supports the long accepted view that butyl alcohol is formed by reduction of butyric acid or a derivative thereof such as butyrylphosphate. The small amount of isotope present in ethyl alcohol and the C_3 compounds is probably the result of side reactions or a limited reversibility of the acetic acid condensation leading to butyric acid.

Labeled acetone was converted exclusively to isopropyl alcohol. The decrease in the isotope content of the acetone during the fermentation can be taken as positive proof that acetone is a normal intermediate in the formation of isopropyl alcohol from carbohydrate.

Two other bacteria, *Butyribacterium rettgeri* and *Cl. kluyveri,* also have been shown to convert acetate to butyrate (14, 54). The latter organism is of particular interest since it utilizes ethyl alcohol and acetate as substrates instead of carbohydrates as do all other butyric acid bacteria, and it forms both caproic and butyric acids. A conversion of C_2 compounds into C_4 and C_6 fatty acids can therefore be directly observed. The formation of caproic acid from ethyl alcohol and acetate has been shown to involve two steps, butyric acid being an intermediate:

(a) $CH_3CH_2OH + CH_3COOH = CH_3(CH_2)_2COOH + H_2O$

(b) $CH_3CH_2OH + CH_3(CH_2)_2COOH = CH_3(CH_2)_4COOH + H_2O.$

When carboxyl-labeled acetate and ordinary ethyl alcohol are supplied, the butyric acid formed contains the isotope almost equally distributed between the carboxyl and beta positions. Caproic acid contains one third of the isotope in the carboxyl group, the rest presumably being in the beta and delta positions, though this is not certain. The isotope content of the residual acetic acid is much lower than that of the added acetic acid. This evidently results from the oxidation of ethyl alcohol to acetic acid or a related compound such as acetylphosphate in isotopic equilibrium with it.

When *Cl. kluyveri* acts upon ordinary ethyl alcohol and carboxyl-labeled butyric acid, the isotope is found in caproic acid but not in acetic acid. Contrary to expectation, the caproic acid contains none of the isotope in the carboxyl position. These results indicate that caproic acid is formed by the condensation of the carboxyl group of butyric acid or butyrylphosphate (28) with the methyl group of a C_2 compound, probably acetic acid, as follows:

$$CH_3(CH_2)_2C^*OOH + CH_3COOH + 4\,H$$
$$= CH_3(CH_2)_2C^*H_2CH_2COOH + 2\,H_2O.$$
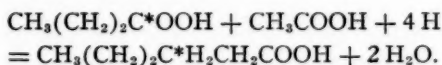
It seems likely that further reactions of this type are involved in the synthesis of the more common C_{16} to C_{18} fatty acids in higher plants and animals (55).

The type of sugar fermentation caused by *Cl. welchii* has been shown to be determined by the adequacy of the iron nutrition (56). Normal cells cause an acetic-butyric acid type of fermentation in which not over 20 per cent of the sugar is converted to lactic acid. Iron deficient cells, on the contrary, produce very little volatile acid but convert up to 85 per cent of the sugar to lactic acid. It appears from this work and the earlier experiments of Kempner & Kubowitz[2] on inhibition of the butyric fermentation by carbon monoxide and cyanide that an iron enzyme is involved in the decomposition of pyruvate to carbon dioxide, hydrogen, and acetic acid. In the absence of sufficient iron, pyruvate accumulates and is reduced to lactate.

The relatively stable acetoacetic acid decarboxylase of *Cl. acetobutylicum* has been partially purified and its properties studied (57). The enzyme appears to be highly specific; no substrate other than acetoacetate is known to be attacked. In dilute solution the enzyme is dissociated but the component parts have not been separated. There

[2] *Ann. Rev. Biochem.*, **4**, 603–4 (1935).

is some evidence however that riboflavin phosphate may be the prosthetic group.

Mixed acid and butanediol fermentations.—Much progress has been made in our knowledge of the conversion of pyruvate to acetate and formate by *E. coli*. This reaction, long thought to involve a hydrolytic cleavage of pyruvate, was called the "hydroclastic reaction." Recent studies with cell-free enzyme preparations (58) have shown that inorganic phosphate rather than water enters into the reaction which should therefore more properly be called the "phosphoroclastic reaction" (59). The rate of pyruvate decomposition is dependent upon the concentration of inorganic phosphate, the maximum rate being attained at about $0.02\ M$. During the reaction inorganic phosphate disappears and formate and a labile phosphate compound, resembling acetylphosphate, accumulate (29). The quantitative data are essentially in agreement with the equation:

$$CH_3COCOOH + H_3PO_4 \rightleftharpoons CH_3COOPO_3H_2 + HCOOH.$$
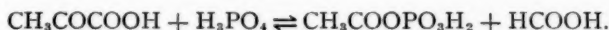
If adenylic acid or glucose is added, the labile phosphate is transferred to these acceptors. The reversibility of the above reaction has been established in several ways. (*a*) Pyruvate has been detected in small amounts as a product of a reaction between acetylphosphate and formate (7). (*b*) When ordinary pyruvate is partially decomposed in the presence of $HC*OOH$, $CH_3COC*OOH$ is formed (8, 60). The carboxyl groups of the two labeled compounds eventually reach isotopic equilibrium as would be expected. The possibility that carbon dioxide is an intermediate in the exchange of labeled carbon between formate and pyruvate is excluded experimentally. (*c*) When ordinary pyruvate is broken down in the presence of CH_3C*OOH and adenylpyrophosphate, the residual pyruvate is labeled in the carbonyl group (8). (*d*) A rapid exchange of radioactive phosphate occurs between inorganic phosphate and acetylphosphate as would be expected from the above reaction (7). From approximate equilibrium data, it is calculated that the equilibrium constant for the condensation reaction is of the order of 0.01. The ΔF_0 is therefore about 2 to 3 Kcal.

The formation of succinate by cell-free preparations of *E. coli* evidently occurs by two distinct mechanisms, one involving carbon dioxide fixation, the other a condensation of acetic acid (61). Succinate formation equivalent to the carbon dioxide uptake can occur during anaerobic pyruvate decomposition in an atmosphere of 5 to 10 per cent carbon dioxide in hydrogen. If labeled carbon dioxide is used

the isotope becomes incorporated in the carboxyl groups of succinate, lactate, and formate. The isotope content of succinate is consistently lower than would be expected if all of it were formed from carbon dioxide. The existence of a second mechanism of succinate formation was demonstrated by fermenting pyruvate in the presence of carboxyl-labeled acetate; the resulting succinate was labeled in the carboxyl groups. On the basis of the relative isotope contents of acetate and succinate at the end of the fermentation, it is estimated that from 10 to 40 per cent of the succinate is formed from acetate.

Cell suspensions of *Aerobacter indologenes* catalyze two distinct types of acetate condensation, one involving the methyl groups, the other the carboxyl groups (10). When carboxyl-labeled acetate is supplied to cells fermenting glucose, carboxyl-labeled succinate is formed, indicating that the condensation occurs through the methyl group of acetic acid. It is not clear whether two molecules of acetate condense directly as the authors suggest, or whether a more complex mechanism is involved (62). The second type of acetate condensation involving the carboxyl group leads to the formation of 2,3-butyleneglycol. It is suggested without direct evidence that acetaldehyde is an intermediate in this reaction. In view of later work on the reversibility of the phosphoroclastic decomposition of pyruvate, it is possible that pyruvate rather than acetaldehyde is the actual intermediate (8). Two other reactions catalyzed by *A. indologenes* are the reduction of acetate to ethyl alcohol and the conversion of succinate to acetate. All the above reactions occur to a quantitatively significant extent.

Yellow and orange pigment strains of *Escherichia* and *Aerobacter* decompose sugar in the same way as nonpigmented strains (63).

There has been a renewed interest in fermentations giving high yields of 2,3-butanediol because of the usefulness of this compound in the preparation of synthetic rubber. Quantitative determinations of the products of glucose, xylose, and pyruvate fermentations by *Aeromonas (Proteus) hydrophila* and *Aerobacillus polymyxa* demonstrate a great similarity to *Aerobacter* fermentations (64, 65). The decomposition of xylose by these organisms cannot be explained on the basis of a simple $C_3 - C_2$ splitting of the C_5 chain; in this respect it resembles a number of other bacterial fermentations of pentoses. *Aerobacter, Aerobacillus,* and *Aeromonas* fermentations differ most when pyruvate is the substrate; all three organisms form acetate, carbon dioxide, and hydrogen, but as a fourth product *Aerobacter* gives 2,3-butanediol, *Aerobacillus* forms acetoin, and *Aeromonas* produces

lactic acid. The yields of butanediol and other products vary greatly with the structure and state of oxidation of the substrate. With *Aerobacillus,* for example, the yield of butanediol in moles per 100 moles of substrate fermented is 65 with glucose, 38 with xylose, 14 with mannitol, and 0 with pyruvate (65). Many *A. polymyxa* strains are susceptible to inhibition by streptothricin and bacteriophage, but there appears to be no relation between susceptibility and the yield of butanediol (66, 67).

There are three stereoisomers of 2,3-butanediol, the meso, dextro, and levorotatory forms. The two latter have been shown to be $l(+)$ and $d(-)$, respectively (68). It is worth noting here that acetylmethylcarbinols and butanediols that show the same sign of rotation also possess the same spatial configuration (69). Bacteria differ with respect to the butanediol isomers formed. *Aerobacillus polymyxa* produces only $d(-)$-butanediol which has a specific rotation $[\alpha]_D^{26} = -13.34°$ (70, 71); *Aerobacter aerogenes* forms a mixture of isomers consisting mainly of meso- with a little $l(+)$-butanediol (34, 71); *Aeromonas hydrophila* produces the $d(-)$ and meso isomers, the latter predominating (64); *Bacillus subtilis* gives an equal mixture of $d(-)$ and meso isomers and in addition forms a considerable amount of glycerol (70).

The anaerobic catabolism of *Pasteurella pestis* is of the mixed acid type (36); however, the high yield of acetate and ethyl alcohol and the low yield of C_1 compounds indicate that a synthesis of acetic acid from carbon dioxide may also occur.

Other fermentations of non-nitrogenous substrates.—It has been known for some time that lactic acid production from glycerol by most species of *Streptococcus, Lactobacillus,* and *Staphylococcus* occurs slowly and irregularly. The essential factor controlling the process is now known to be the oxygen supply (33, 72, 73). Lactic acid bacteria can be divided into two groups with respect to glycerol fermentation. Members of the larger group ferment glycerol only when oxygen is supplied. A few species in the smaller group, including *Lactobacillus lactis* and some enterococci, can utilize glycerol either aerobically or anaerobically; in the absence of oxygen an organic hydrogen acceptor like fumarate is used. The function of oxygen or the organic hydrogen acceptor is to oxidize glycerophosphate to triosephosphate. The further conversion of triosephosphate to lactic acid does not require an outside oxidizing agent.

Homofermentative lactic acid bacteria, both streptococci and lacto-

bacilli, can use citrate as a carbon and energy source (74, 75). In this respect they differ from the heterofermentative species that decompose citrate in growing cultures only when sugar is also supplied. The products of citrate fermentation by *S. faecalis* at pH 8.5 are mainly acetate, carbon dioxide, and formate in the molecular ratio of 2 : 1 : 1. As the pH of the culture medium is lowered to 5.0, acetate and formate decrease, carbon dioxide increases, and a considerable quantity of lactate and a little acetoin are formed. Growth is almost equally good over the pH range 5.0 to 8.5 showing that the same or similar energy-yielding mechanisms must be functioning over the entire range. The results are consistent with the following schematic mechanism of citrate decomposition:

$$\text{citrate} \rightarrow \text{acetate} + \text{oxaloacetate} \rightarrow CO_2$$
$$+ \text{pyruvate} \rightarrow \text{acetate} + \text{formate}.$$
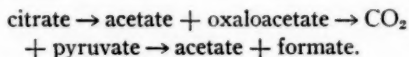
The main secondary reaction, under acid conditions, is evidently a dismutation of pyruvate.

Several enzymes concerned in the conversion of glucose to lactic acid were demonstrated in dried and cell-free preparations of *A. aerogenes, S. albus,* and *S. lutea* (76, 77, 78). Evidence is presented for the formation of α-keto, γ-hydroxy valeric acid by *S. albus* from pyruvate under aerobic conditions.

In the conversion of glucose to lactate by *Lactobacillus casei,* the carboxyl group of lactate is derived from the third and fourth carbon atoms of glucose; this was demonstrated by the use of C^{13} (79). The absence of labeled carbon from the methyl group of lactic acid supports the generally accepted view that no symmetrical C_3 compound is involved in the formation of lactate.

A recently described cellulose-fermenting bacterium, *Clostridium cellobioparus,* is able to attack a much greater variety of carbohydrates, including glucose and cellobiose, than previously studied species (80). The products of glucose and cellulose fermentation include ethyl alcohol, lactate, formate, acetate, carbon dioxide, and hydrogen; 30 to 40 per cent of the substrate is evidently converted to nonacid products that could not be identified. Cellobiose but not glucose accumulates under certain conditions as a product of cellulose decomposition.

A new approach to the problem of bacterial cellulose decomposition has been introduced by the demonstration that both aerobic and anaerobic bacteria attack the cellulosic components of plants much more readily than pure cellulose preparations like filter paper (81, 82,

83). Indeed, certain organisms, like *Clostridium roseum* and *Bacillus aporrhoeus,* that appear not to attack isolated cellulose at all, decompose plant tissue cellulose vigorously. It is evident that the presence of pectins, xylans, and other polysaccharides in plant tissues greatly facilitates the decomposition of the closely associated cellulose.

There is considerable evidence that α-hydroxy acids are not intermediates in the interconversion of fatty and α-keto acids. This idea is further supported by the observation that the reduction of pyruvate by *Propionibacterium pentosaceum* occurs readily in the presence of sufficient fluoride to inhibit completely the utilization of lactate and yet no lactate accumulates (84). The true mechanism of pyruvate reduction is still unknown.

Fermentations of nitrogenous compounds.—The list of amino acids that can serve as carbon and energy sources for anaerobic growth has been extended by the discovery of bacteria capable of fermenting glycine and alanine (85). The generalization that amino acids cannot be fermented unless they contain substituent groups (85a) is therefore no longer valid in all cases. Alanine undergoes a typical propionic acid type of fermentation under the influence of an unnamed anaerobic spore-former:

$$3 \, CH_3CHNH_2COOH + 2 \, H_2O = 3 \, NH_3$$
$$+ \, 2 \, CH_3CH_2COOH + CH_3COOH + CO_2.$$
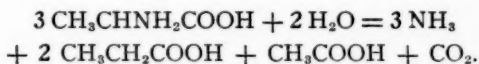
Lactate, pyruvate, and serine are similarly fermented. Threonine, however, gives both butyric and propionic acids as the characteristic product:

$$3 \, CH_3CHOHCHNH_2COOH + H_2O = 3 \, NH_3$$
$$+ \, CH_3CH_2CH_2COOH + 2 \, CH_3CH_2COOH + 2 \, CO_2.$$
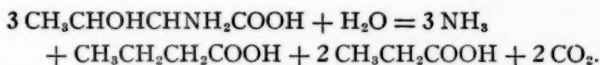
Glycine is fermented readily by an unnamed Gram-positive anaerobic coccus that attacks other amino acids slowly or not at all. The approximate equation for glycine fermentation is:

$$4 \, CH_2NH_2COOH + 2 \, H_2O = 4 \, NH_3 + 3 \, CH_3COOH + 2 \, CO_2.$$
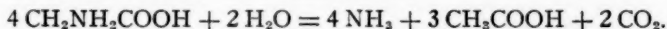
Under some conditions gaseous hydrogen is also formed.

Unlike the above mentioned organisms, *Cl. tetani* cannot utilize the simple alpha amino acids as energy sources, but ferments glutamic and aspartic acids, histidine, serine, and probably threonine with the production of ammonia, carbon dioxide, acetic, and butyric acids (86, 87). Several nonnitrogenous compounds (malate, fumarate, pyruvate)

can also be used as anaerobic energy sources. It may be noted here that *Acetobacter suboxydans,* a typical aerobe, is able to deaminate serine anaerobically (88).

One of the lactic acid bacteria, *Streptococcus allantoicus,* has been shown to be able to ferment a nitrogenous compound, allantoin, as well as sugar (89). The products are ammonia, urea, oxamic acid, carbon dioxide, formic, acetic and lactic acids, and possibly glycolic acid. Oxamic acid had not previously been reported as a product of bacterial metabolism. *E. coli, A. aerogenes,* and *P. vulgaris* can also utilize allantoin slowly in a complex medium, probably as a secondary nitrogen source (90).

It has been known for some time that serine, in contrast to alanine, can be deaminated under anaerobic conditions by *E. coli.* Further work with *E. coli, Ps. pyocyaneus, Proteus OX-19,* and *Cl. welchii* has shown that whereas both *d-* and *l-*serine are rapidly deaminated, various hydroxy substituted derivatives are not attacked (91). The main products of serine decomposition under appropriate conditions are ammonia and pyruvic acid. Threonine undergoes a similar reaction to give α-keto butyric acid. The first step in these reactions is thought to be the removal of water from the alpha and beta carbon atoms under the influence of a dehydrating enzyme or dehydrase similar to enolase, followed by a nonenzymatic rearrangement and hydration to give the corresponding α-keto acid plus ammonia:

$$HOCH_2CHNH_2COOH \xrightarrow{-H_2O} CH_2 = C(NH_2)COOH \rightarrow$$
$$CH_3C(NH)COOH \xrightarrow{+H_2O} CH_3COCOOH + NH_3.$$
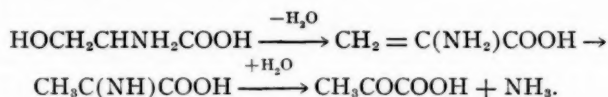
The postulated intermediates are as yet hypothetical. Crude cell-free preparations of serine deaminase are inactivated by dialysis and can be reactivated by addition of the ions of zinc, magnesium, and manganese. Fluoride and cyanide are powerful inhibitors (92).

Cysteine is decomposed by *E. coli* and *Bac. subtilis* to give hydrogen sulfide, ammonia, and pyruvate (93 to 98). With *E. coli,* hydrogen sulfide and ammonia are always formed in a 1 : 1 ratio even in the presence of various inhibitors (cyanide, pyruvate, or oxygen). This leads to the view that the two compounds are formed under the influence of a single enzyme variously called cysteinase and desulfurase (92). S-methyl-(*l*)-cysteine, N-methyl-(*l*)-cysteine, isocysteine, and glutathione are not appreciably decomposed but glycyl-(*l*)-cysteine

and (l)-cysteinylglycine yield hydrogen sulfide and ammonia probably after preliminary hydrolysis (99). All evidence now available is consistent with the view that the mechanism of cysteine decomposition is similar to that of serine and involves a primary removal of hydrogen sulfide:

$$HSCH_2CHNH_2COOH \rightarrow CH_2 = CNH_2COOH + H_2S.$$
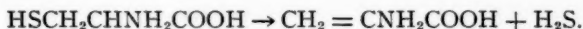
Like serine deaminase, desulfurase is activated by metallic ions.

The reduction of trimethylamine oxide to trimethylamine is catalyzed by most bacteria of the family *Enterobacteriaceae* (100).

A large number of substances, both nitrogenous and non-nitrogenous, can be dehydrogenated by *Cl. botulinum, Cl. parabotulinum,* and *Cl. welchii* (101); the compounds attacked most rapidly are ethyl alcohol, alanine, and leucine. The individual dehydrogenases are differently affected by various reagents like cyanide, azide, and arsenate.

BACTERIAL ENZYMES

Respiratory enzymes.—An active and highly specific enzyme preparation which catalyzes the oxidation of pyruvate to acetate and carbon dioxide, has been obtained from *Proteus vulgaris* (30). The enzyme appears to be a complex of specific protein, diphosphothiamine, and a bivalent metallic ion. Normally the metal is probably magnesium, although manganese, iron, cobalt, nickel, or zinc can be substituted. The enzyme is reversibly inhibited by pyrophosphate, which appears to compete with diphosphothiamine.

Propionibacterium pentosaceum contains cytochromes-*a* and *b*, but not *c*. It appears to possess a cytochrome oxidase which presumably mediates between cytochrome-*a* and oxygen and is insensitive to cyanide and hydrogen sulfide. The oxidase activity is inhibited by carbon monoxide, but the inhibition is not reversed by light (102). A somewhat similar cytochrome system is also present in *Bacillus subtilis* (95).

The respiration of *Bac. subtilis* and *Pseudomonas aeruginosa* is inhibited by sodium azide, which is known to poison heme systems. The addition of pyocyanine to the medium of a pyocyanine-free strain of *P. aeruginosa* counteracts this effect, presumably by providing an alternative hydrogen carrying system which can react with oxygen (103). Azide also inhibits bacterial catalase (104).

New light has been thrown on the utilization and synthesis of iron porphyrins by *Hemophilus influenzae* (105). This organism can

insert iron only into porphyrins which contain vinyl groups, although it can use some vinyl-free iron porphyrins if these are supplied. Iron-free porphyrins lacking vinyl groups inhibit the utilization of iron porphyrins, apparently by competing with them for the specific proteins of heme enzymes. The porphyrin-protein combination may involve the interaction of the ionized propionic acid side chains of the porphyrin with basic groups of the protein, since esterification destroys both the catalytic and inhibitory properties of the porphyrins. Vinyl groups appear to be necessary also for the reduction of nitrate to nitrite, since bacteria grown with vinyl-free iron porphyrins do not carry out this reaction.

Since *Streptococcus mastiditis* can oxidize alcohol without hydrogen peroxide formation, it presumably possesses a peroxidase (32). This enzyme, however, is relatively insensitive to cyanide as compared to heme systems and the peroxidase of *Acetobacter peroxidans.*

By analogy with *Cypridina* luminescence, bacterial luminescence is believed to be due to the aerobic oxidation of a coenzyme, luciferin, which serves as a hydrogen carrier in the respiratory system of the organism, under the influence of the enzyme luciferase. Although neither luciferin nor luciferase has yet been obtained in cell-free preparations from luminous bacteria, an interesting study on the inhibition of luminescence in *Photobacterium phosphoreum* by irradiation with blue light has given an indication of the nature of bacterial luciferin (106). The inhibition is probably due to photochemical inactivation of dehydroluciferin. A study of the inactivation spectrum indicates that this substance may be a vitamin K derivative. This structure seems more consistent with the available data on *Cypridina* luciferin than is the coenzyme-flavin structure recently proposed (107, 108). An interesting study of the quinine inhibition of bacterial luminescence (109) has been added to the already impressive list of experiments on the nature and control of reactions in bioluminescence, which have been recently and comprehensively reviewed (110).

Some bacteria can use tetrathionate as an alternative hydrogen acceptor for oxygen, by reducing it to thiosulfate (111, 112). The "tetrathionase" of *Salmonella paratyphi* appears to be an adaptive enzyme which can be produced in the absence of an added source of nitrogen or growth of the organisms (113).

Enzymes involved in amino acid decomposition.—Many bacteria possess enzymes which catalyze the decarboxylation of certain amino acids to the corresponding amines. Six types of amino acid decar-

boxylases have been found to date, and extensive studies have been carried out on partially purified preparations of each type. The enzymes are quite specific with regard to substrates; $l(+)$lysine, $l(-)$-tyrosine, $l(-)$histidine, $l(+)$arginine, $l(+)$ornithine, and $l(+)$glutamic acid are decarboxylated by different enzymes. While some organisms possess only one or two decarboxylases, others may contain a number of such enzymes. Different strains of the same species may vary in their characteristic complement of decarboxylases. Frequently, active preparations can be obtained from dried acetone powders of bacteria, and the enzymes purified by adsorption and elution, followed by fractional precipitation with ammonium sulfate. The proper choice of organisms and specific differences in the stability of the different enzymes have aided in the separation of the decarboxylases. With two exceptions, the amino acid decarboxylases can each be readily resolved into an apoenzyme and a coenzyme. The coenzyme is widely distributed in living tissues (114), and appears to be common to all of the resolvable decarboxylases so far investigated. The natural coenzyme is probably a phosphorylated derivative of pyridoxal, since it can be replaced in enzyme preparations either with pyridoxal together with ATP or with synthetic coenzyme prepared by chemically phosphorylating pyridoxal with phosphorus oxychloride, $POCl_3$ (115 to 118). On hydrolysis, the natural coenzyme yields material which behaves like pyridoxal.

Specific preparations of tyrosine decarboxylase have been obtained from *Streptococcus fecalis* (119). Unlike mammalian decarboxylase, the bacterial enzyme attacks, in addition to tyrosine, $l(-)$3,4-dihydroxyphenylalanine. The enzyme dissociates spontaneously during purification, and more completely on alkaline ammonium sulfate precipitation or dialysis. Streptococci grown in pyridoxine-deficient media produce the apoenzyme, but show poor decarboxylase activity (117, 120). Suitable media have been described for the growth of cells for decarboxylase and apoenzyme production, respectively (121, 122).

Lysine decarboxylase has been obtained from *E. coli* and *B. cadaveris* (123). $l(+)$Lysine could not be replaced as substrate by any other amino acid tested or by acetylated or methylated lysine derivatives. Ornithine decarboxylase preparations from *Cl. septicum* and arginine decarboxylase from *E. coli* are also specific for the respective amino acids (124). All three of the above decarboxylases may be resolved into apoenzymes and coenzymes.

Although the glutamic acid decarboxylase from a strain of *E. coli* requires coenzyme for its activity (118), the enzyme obtained from another coliform organism could neither be resolved nor used as source of coenzyme for the tyrosine decarboxylase system, indicating either the absence of coenzyme or its very close association with the protein (124).

Partially purified histidine decarboxylase from *Cl. welchii* also could not be resolved (125). The pH optimum of the enzyme (pH 4.5) differs considerably from the optimal pH at which the intact cells carry out the reaction (pH 2.5). This is undoubtedly due to the difference between internal and external pH of intact cells. Acetyl- and benzoyl-histidine are not attacked by the histidine decarboxylase of *E. coli* (126).

The use of bacterial decarboxylases for the assay of amino acids with the manometric technique has given very satisfactory results (127, 128).

The ability of bacteria to carry out transaminations at high rates has been shown in studies with washed cells of a number of species. The following reaction was studied: $l(+)$glutamic acid $+$ oxaloacetic acid $\rightarrow$ α-ketoglutaric acid $+$ $l(-)$aspartic acid (129).

In experiments with cells of *S. faecalis* grown in pyridoxine-deficient media and with resolved cell-free transaminase preparations from normal cells, it has been shown that synthetic "pyridoxal phosphate" can serve as coenzyme for transaminase activity. Pyridoxal with ATP is also effective in some cases (129a).

Proteus vulgaris is able to oxidize practically all the known natural amino acids. These oxidations evidently involve several distinct amino acid oxidases differing in stability. The most stable of these enzymes, which can be obtained cell-free by disintegrating the bacteria with supersonic vibrations, is an *l*-amino acid oxidase (130). It oxidizes the *l*-forms of phenylalanine, norleucine, and leucine and eight other amino acids quantitatively to the corresponding alpha keto acids. Unlike the *l*-amino acid oxidase of rat kidney, this enzyme does not form hydrogen peroxide. In cell-free preparations it is associated with insoluble particles that can be sedimented effectively only in gravitational fields higher than $3000 \times g$. This enzyme should be useful for preparing *d*-amino acids and the corresponding keto acids.

Acetobacter suboxydans deaminates a number of amino acids aerobically (88). It is noteworthy that the two most essential amino acids as determined by growth experiments, valine and isoleucine, are deam-

inated very slowly if at all. The nonessential and stimulating amino acids, on the contrary, are rather rapidly deaminated. This suggests that the essential amino acids are used as such while the nonessential amino acids serve primarily as sources of ammonia.

Bac. brevis evidently does not contain the *l*-amino acid oxidase of *Proteus vulgaris* since some of the amino acids oxidized most rapidly by the latter are not attacked by the former (131). The amino acids rapidly deaminated by *Bac. brevis* are alanine, glutamic and aspartic acids, serine, and arginine.

Asparaginase has been found in a number of bacteria and certain properties of cell-free preparations of the enzyme have been studied (132).

Enzymes involved in carbohydrate decomposition and synthesis.— Amylases obtained from various bacteria differ widely in their starch-degrading properties. While some resemble malt alpha amylase in exhibiting strong dextrinizing and weak saccharifying power, others cause more or less complete breakdown of starch to maltose (135). The amylase of *Clostridium acetobutylicum* leaves in the medium no stable by-products other than maltose. It can be concentrated and rendered almost free of maltase activity by adsorption on starch (136). When the same organism is grown with maltose as substrate, maltase preparations free of amylase can be obtained. This bacterial maltase differs from most maltases in having a low pH optimum (4.5). It is a specific maltase and not merely an α-glucosidase, since it does not attack sucrose.

The enzyme of *Bacillus macerans,* which produces Schardinger dextrins from starch, has been used to study the properties of different starches and of starch fractions (137).

Phosphorolytic degradations differ from hydrolyses in that phosphoric acid replaces water in the reaction. The phosphorolysis of sucrose has been shown with *Leuconostoc mesenterioides* (138) and *Pseudomonas saccharophila* (40). Preparations of the adaptive enzyme sucrose phosphorylase have been obtained from dried cells of the latter organism and rendered virtually free of invertase and phosphatase activity by ammonium sulfate precipitation (41). This enzyme catalyzes the reversible reaction:

$$\text{sucrose} + \text{phosphate} \rightleftharpoons \text{glucose-1-phosphate} + \text{fructose}.$$
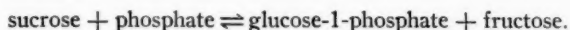
With the aid of this enzyme, the synthesis of sucrose has been accomplished for the first time (139). Two new analogues of sucrose, α-*d*-

glucosido-α-*l*-sorboside and α-*d*-glucosido-β-*d*-ketoxyloside, were prepared with the same enzyme by substituting *l*-sorbose and *d*-ketoxylose for fructose in the reaction from right to left (140, 141). The discovery of the phosphorolytic breakdown and synthesis of sucrose not only emphasizes the importance of phosphoric esters in biological condensation reactions, but also throws some light on the structure of sucrose and on the transformation of fructose in the formation of fructosides (142). Sucrose phosphorylase does not contain an easily dissociable coenzyme. The enzyme is insensitive to fluoride, but is strongly inhibited by glucose which appears to compete mainly with sucrose for a position on the enzyme. The occurrence of phosphorolysis of sucrose could be demonstrated *in vivo* as well as *in vitro;* but no evidence for a phosphorolytic breakdown of other natural disaccharides could be found (42). The initial steps in the breakdown of raffinose appear to be a hydrolytic cleavage of the trisaccharide by an intracellular melibiase, followed by phosphorolysis of the sucrose thus formed.

Still another type of sucrose decomposition by certain bacteria involves the formation of polysaccharides from either the glucose or fructose portion of the sucrose molecule by what appears to be a direct exchange of glycosidic bonds. An enzyme obtained from cultures of *Leuconostoc mesenterioides* causes the liberation of fructose by the exchange of the sucrose linkage for a glucosidic bond of the polymer, dextran, in accordance with the equation:

$$\text{n } C_{12}H_{22}O_{11} \rightarrow \text{n } C_6H_{12}O_6 + (C_6H_{10}O_5)_n.$$
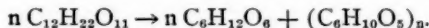
No intermediate accumulation of a phosphoric ester of glucose appears to be involved (143). The separation of the enzyme from the viscous polysaccharide in the medium was accomplished by adsorption on a chloroform emulsion followed by alcohol precipitation. The dextran produced *in vitro* was shown to be immunologically active and identical with that produced *in vivo* (144).

A fairly large variety of bacteria can produce the polysaccharide levan from sucrose or raffinose with the liberation of free glucose or melibiose, respectively. The immunological identity of such levans formed by cell-free enzyme preparations of different bacteria indicates their chemical similarity (145, 146). Crude preparations of levan-synthesizing enzymes invariably cause the production of fructose in addition to levan and glucose from sucrose. While it seems very probable that a single enzyme is involved in the polysaccharide synthesis,

it is as yet impossible to decide whether the same or another enzyme is responsible for the inversion. Extensive studies with enzyme preparations obtained from the cells of *Aerobacter levanicum* have revealed some interesting and as yet unexplained features of the reactions involved (147 to 150). It is quite clear that no appreciable accumulation of a phosphoric ester precedes levan synthesis and that glucose has an inhibitory effect on this process. Analysis of the data is complicated by the large number of variables which must be taken into consideration. These include not only the synthetic and hydrolytic products from the substrate, but also the method of preparation of the enzyme and its inactivation during the reaction. Although attempts to demonstrate the reversible nature of the levan-forming reaction have not been successful with preparations from *A. levanicum*, indirect evidence of the reversibility of the process has been obtained with the extracellular enzyme of *Bacillus subtilis* (151). In a system containing bacterial enzymes, levan, and yeast invertase, the addition of glucose was shown to increase the rate of reducing sugar formation from the polysaccharide, presumably as a result of sucrose formation through a direct reaction between levan and glucose.

Some bacteria produce rather large amounts of capsular or soluble polysaccharides from substrates other than sucrose. In many organisms such as *Acetobacter xylinum,* the polysaccharide formation does not depend on the presence of specific sugars. On the other hand, *Phytomonas tumefaciens* (152) produces appreciable quantities of soluble glucosan from glucose, fructose, or sucrose, but only small amounts from xylose or arabinose. This indicates that although interconversion of the sugars can occur, the precursor of the polysaccharide, which may well be glucose-1-phosphate, is formed most readily from hexose substrates.

Miscellaneous enzymes.—The ribonuclease activity of *Pasteurella pestis* can be demonstrated with intact or killed cells as well as with cell-free preparations (153). Only part of the nucleic acid is hydrolyzed by such preparations to mononucleotides, the remainder probably existing in a partially depolymerized state. Only a trace of phosphate is liberated. The depolymerase of the complex is most stable to heat inactivation at pH 6.5, while the tetranucleotidase and mononucleotidase are more stable at pH 7.6.

The lecithinase activity present in culture filtrates of *Vibrio comma* and of the El Tor vibrio, results in the production of phosphate, choline, and fatty acids from lecithin and is stimulated by calcium or mag-

nesium ions (154). Cholinesterase is present in type I pneumococcus (133). Cell-free preparations of histamine- and nicotine-oxidizing enzymes could not be obtained from soil bacteria (134).

Cell-free plasma coagulase can be obtained from the culture medium in which *Staphylococcus aureus* has been allowed to grow in the presence of plasma (155). Coagulase is believed to be a precursor of a thrombin-like substance, the production of which depends on the participation of an activator, which is present in some, but not all, plasmas (156). Unlike coagulase, the thrombin-like substance is thermolabile.

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IMMUNOCHEMISTRY

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Progress in immunochemistry since the last survey of the field in 1942 has been very extensive. Limitations of space have made it impossible to include in the present review many papers published during this period. Fortunately, several books and a number of reviews on various broad and specialized subdivisions of the field have appeared and may facilitate location of papers not mentioned here. The second edition of Landsteiner's monumental book (1) offers complete coverage of the field up to his untimely death in 1943. Books by Dubos (2), Boyd (3), and Sevag (4) also contain much information and outline a variety of perspectives. This reviewer's opinions on immunochemistry of the proteins have already been given (5) and much of the material covered therein will not be repeated; for an independent evaluation see (6). Other surveys include reviews on immunology in 1943 (7), on the nature of the forces between antigen and antibody (8), on nutrition and antibody formation and on the anamnestic reaction (9, 10, 11), complement (12), brucellosis (13), bacillary dysentery (14), meningococcic meningitis (15), *Salmonellae* (16), the Vi antigen (17), hemolytic streptococci (18), tuberculin (19), and the Wassermann antigen (20); there are also reviews on biologic false positive serological tests for syphilis (21), on immunity in malaria (22), the human blood group substances (23, 24), cold hemagglutination (25); and reviews on allergy (26), the chemistry of pollen allergens (27) and poison ivy extracts (28), and on histamine in anaphylaxis (29, 30).

Quantitative methods and their applications.—The superiority of the quantitative absolute methods for the determination of antibodies, antigens, and complement for immunochemical investigations has become apparent to numerous workers, as evidenced by an increasing proportion of papers in which these techniques are employed. Brief summaries of the range of applicability of these methods have been given by Kabat (5) and Treffers (6).

Schramm & Friedrich-Freksa (31), Kleczkowski (32), and Beale & Lojkin (33) have studied the reaction between tobacco mosaic virus and its homologous antibody by the quantitative precipitin

method and found it to behave like a single antigen-antibody system. As would be expected from the high molecular weight of the virus the ratios of antibody nitrogen to antigen nitrogen in the precipitates were lower than have been obtained with smaller antigens. Molecular ratios of antibody to virus of 600 and 300 to 1 in the precipitate in the region of extreme antibody excess and at the equivalence zone were calculated (31), assuming 23,000,000 as the molecular weight of to-bacco mosaic virus; recalculation of the data with the more reliable value of 33,000,000 for the molecular weight of the antigen gives values of 900 and 450 for the molecular proportions at these points (34). The validity of one set of results (33) is questionable because of the very low antibody content of the sera. Boyd & Malkiel (35) were able to obtain sufficiently strong antisera to crystalline horse hemoglobin using adjuvants containing a suspension of Monilia to study the composition of hemoglobin-antihemoglobin precipitates by nitrogen and iron analyses. Contrary to an earlier report (36) the specific precipitate was soluble in excess antigen. Studies by Hauro-witz et al. (37) have shown that bovine and human hemoglobins are related, since addition of one hemoglobin inhibits precipitation of the other hemoglobin with its homologous antibody.

Haurowitz & Schwerin (38) prepared antisera to complex anti-gens containing two artificially introduced groups such as iodo-arsanil-azo-globulin, and from quantitative measurements of the amounts of antibody removed in sequence by arsanil-azo-ovalbumin, iodo-oval-bumin, iodo-arsanil-azo-ovalbumin, globulin, and iodo-arsanil-azo-globulin, found that no antibody was formed which reacted with the disubstituted antigen coupled with a heterologous protein (iodo-arsa-nil-azo-ovalbumin) after absorption with arsanil-azo-ovalbumin and iodo-ovalbumin. Part of the antibody was specific for the original antigen since it could not be absorbed by other substances.

Quantitative precipitin analyses have been used to study the im-munochemical relationships between native, denatured (with guanidine hydrochloride), and regenerated normal horse globulins and antibodies (39). In confirmation of earlier work (40, 41) native and denatured antibodies were found more closely related antigenically to each other than to native and denatured fractions of normal globulin. Irreversi-bly denatured antibody dissolved in sodium thiocyanate remained serologically active (39); changes in combining ratios and in propor-tion of specifically precipitable nitrogen in regenerated antibodies sug-gest complex formation with non-antibody protein (42, 43, 44). Inacti-

vation of diphtheria antitoxin by urea, as measured by neutralization tests, was independent of the concentration of antitoxin. Urea-denatured antitoxin, however, yielded more precipitate when toxin was added than did native antitoxin, probably as a result of complex formation with other non-antibody proteins (42, 43) as had been noted with acidified antipneumococcal rabbit sera (44) ; for studies on effects of urea on complex formation involving anti-egg albumin see Campbell & Cushing (45). MacPherson & Heidelberger (46) found that crystalline egg albumins denatured by treatment with heat, acid, alkali, or water under controlled conditions behaved identically in their reaction with antisera throughout the region of antibody excess; in the region of antigen excess differences attributable to the degree of aggregation were noted.

Further quantitative studies on the cross-reaction between types III and VIII pneumococcal polysaccharides in horse antisera (47) have made it possible to interpret the cross reaction in terms of the known similarities and differences in chemical composition between the two carbohydrates and to arrive at values of 62,000 and 140,000 for their minimum molecular weights. Three different kinds of type III and type VIII anticarbohydrates were identified, two of which participated in the cross-reaction. The third was strictly type-specific. The relationship between chemical constitution and immunological specificity was further substantiated by the finding of Heidelberger & Hobby (48) that cellulose oxidized by nitrogen tetroxide and containing cellobiuronic acid separated by glucose residues cross reacted with both types III and VIII antipneumococcal horse sera. As was anticipated the oxidized cellulose removed most of the cross-reacting antibody from type III and type VIII antiserum. This cross reaction with types III and VIII antipneumococcal sera could be used to follow the disappearance of intravenously injected oxidized cellulose from the blood stream and its appearance in the urine (49) and also the slow elimination of oxidized cellulose gauze used as packing in operative procedures (50).

A study by Mayer & Heidelberger (51) of the competition between type III and type VIII polysaccharides (S III and S VIII) for the cross-reacting fraction of type VIII antipneumococcal horse serum showed that chemical combination of S III with the cross-reacting fraction of anti-S VIII and of S VIII with anti-S VIII was at least 90 per cent complete in less than three seconds at 0°C. Subsequent aggregation with the formation of insoluble compounds took

place with progressively diminishing velocity. By the addition of homologous polysaccharide during the early stages of the reaction, the cross-reacting S III may be liberated from combination with antibody.

The response of rabbits to intensive immunization with polyvalent pneumococcal vaccines was studied by Bjorneboe (52, 53). In most instances, the rise in antibody protein corresponded to the increase in globulin, but in some animals, an increase was noted in globulin not attributable to antibody to the antigens injected (cf. 54). The protective power of rabbit antisera to *Hemophilus influenzae*, type B, could be completely abolished by specific removal of the anticarbohydrate (55); for protection tests to this organism in the developing chick embryo see Weil & Johnson (56), indicating a half-life of about 70 hours.

Incorporation of an amino acid labeled with N^{15} into the diet of an actively immunized rabbit at a time when the antibody level was declining resulted in the incorporation of N^{15} into the antibody as well as into the other serum proteins and at much the same rate; after administration of the isotope was stopped, the decrease in isotope content of both antibody and the other serum proteins also ran parallel. The half life of an antibody molecule in an actively immunized animal was about two weeks (57). Antibody injected passively into an animal fed N^{15} did not take up any N^{15} and disappeared from the circulation at a much greater rate than did the antibody of an actively immunized animal (58).

The enzyme tyrosinase was found by Adams (59) to be completely precipitated by antibody formed in the rabbit and human, as had been found for other enzyme-antienzyme systems (cf. 5). The enzymatic activity was unaffected by precipitation with antibody.

Quantitative estimation of agglutinin nitrogen has been used by Stokinger, Carpenter & Plack to study cross reactivity among the Neisseria (60). *N. gonorrhoeae* and *N. intracellularis* are more closely related to each other than to *N. catarrhalis* and *N. sicca;* each of eight strains of the gonococcus contain one or more antigens not present in other strains and no classification into types or groups may be made.

By quantitative estimation of the type-specific and group-specific agglutinins and of the type I anticarbohydrate in horse, rabbit, and chicken type I antimeningococcal sera, and assay of the protective power remaining in the supernatant after their removal, Kabat *et al.* (61) were able to show that antibody to the type I polysaccharide is responsible only for a portion of the total protective power of these

antisera. In rabbit and chicken type I antimeningococcal sera, most of the protective antibody is not antipolysaccharide, but antibody to some as yet unidentified type-specific antigen (61). Scherp & Rake (62) were able to remove 87 to 95 per cent of the type I protective antibody from four antimeningococcal horse sera with the type I specific polysaccharide. Unlike antipneumococcal sera, antimeningococcal sera contain a large proportion of group specific antibody (61, 62).

Suspensions of stromata have been used by Heidelberger & Treffers (63) to determine the antibody content of rabbit antisera to sheep erythrocytes. Cohen (64) found that treatment of suspensions of *B. proteus* OX-19 with benzene sulfonylchloride did not alter their capacity to combine with rabbit antisera to untreated organisms since identical quantitative agglutinin curves were obtained with untreated and substituted organisms. Differences in the amount of each suspension required for visible agglutination were noted.

The antigenic composition of edema fluids from guinea pigs infected with *B. anthracis* has been studied by Staub & Grabar (65, 65a, 66). By quantitative precipitin estimations, edema fluid was shown to contain two polysaccharides, one of which was identical with the anthrax carbohydrate previously isolated by Ivanovics. Neither polysaccharide was associated with protection; the antibody to the d-glutamyl polypeptide could also be absorbed without removal of protective antibody. Some evidence for the existence of another factor responsible for protection was obtained (67).

Goebel, Binkley & Perlman (68) followed the activity of various fractions isolated from *Sh. paradysenteriae* (Flexner) at various stages during the purification of the specific antigens by a turbidometric modification of the quantitative precipitin method, and also used this technique to study the stability of the products.

Calibrated rabbit antisera to Bence-Jones protein from the urine of individuals with multiple myeloma have been used to detect and estimate quantitatively the amount of Bence-Jones protein in the serum. Since Bence-Jones proteins from different individuals differ serologically, it is necessary to use serum and urine from the same patient (69).

Two laboratories (70, 71) have shown by quantitative immunochemical methods that considerable amounts of normal tissue constituents are present even in the most highly purified influenza virus preparations obtained from allantoic and amniotic fluids either by differential ultracentrifugation or by adsorption and elution from ery-

throcytes. Comparison of purified fractions obtained by ultracentrifu-
gation from virus-containing tumors and from normal chicken tis-
sues had already provided evidence that the bulk of the purified
material from chicken tumors is immunologically identical with its
analogue from normal chicken tissue (for a summary cf. 5). Since
all other purified animal viruses have been prepared by essentially
similar procedures, and since the absence of large amounts of normal
protein in these materials has not been excluded by immunological
methods, it is doubtful whether any of the analytical data reported for
animal viruses is in any way characteristic or representative of the
virus moiety itself. It is therefore not surprising to find the chemical
composition reported for the animal viruses to be almost identical
with that of comparable fractions of the normal tissues from which
they were derived (cf. 5, table 9). These products, however, appear
to be of considerable value in immunization (72).

The scope and utility of quantitative precipitin methods have been
greatly increased by the introduction of micro methods which permit
the estimation of as little as 2 to 10 µg. of antibody nitrogen with a
precision of ±2 µg. (73, 74). These procedures involve estimation of
the amount of specific precipitate by the color produced with a modi-
fied Folin-Ciocalteu reagent and have proven especially useful for the
estimation of antibodies in human sera (73). Heidelberger & Ander-
son (75) have employed one method (73) to study the immune re-
sponse of human patients to pneumococcal pneumonia terminated by
chemotherapy. Variations in antibody N content from 0 to 125 µg.
per ml. were noted. The same method (73) also served to measure the
antibody response of humans injected with type I meningococcal poly-
saccharide and established that this substance was a relatively poor
antigen (76). The micro procedure was successfully applied to the
estimation of isoantibodies A and B in the sera of normal and im-
munized humans of the four blood groups, using as precipitinogens
purified blood group A and A + B substances obtained from hog and
horse stomachs respectively (77). The precipitin reaction between
blood group A substance and its homologous isoantibody obtained
from the sera of immunized individuals of groups O and B was found
to be of the same general type as other antigen-antibody systems (77).
A modified procedure, in which the antibody N in purified solutions
of Wassermann antibody is measured by the decrease in nitrogen in
the supernatant after specific removal of Wassermann antibody on
lipoid floccules, has been introduced by Davis et al. (78, 79).

In using these micro procedures, it is necessary with many systems to remove complement by addition of an unrelated antigen-antibody system and removal of the specific precipitate so obtained, before carrying out the analyses (73, 75, 76). Egg albumin and rabbit anti-egg albumin are most frequently used for removal of complement (80). For quantitative precipitation of small quantities of antibody in human sera, it is necessary to allow tubes to remain in the refrigerator for a week to ten days and it is therefore essential to take precautions to ensure sterility in setting up the determinations (73, 75, 76, 77).

Pauling *et al.* (81 to 91) have worked extensively with a micro method (74) for the estimation of antibody in their studies on the reaction of simple substances with antibody. They have used the procedure to estimate the precipitating potency of haptens containing two or more reactive groups per molecule (cf. 1) and to measure quantitatively the relative capacity of other simple haptens to inhibit precipitation. Their choice of two days in the refrigerator as the period for carrying out these tests seems unfortunate and detracts from the precision of their results, since other investigators have emphasized the need for using intervals of at least a week in assaying such small amounts of antibody (73, 75). This is indeed confirmed by the work of the Pauling group who reported data (table V in 86) indicating that two days are not adequate for maximum precipitation, even in the region of excess antibody, but they continued to use the shorter period.

Complete information is not yet available about which of the components of complement are actually bound by specific precipitates [for a discussion cf. (5)]. Bovine serum, although containing only C'1, has been found by Bier (92) to contain amounts of complement-combining nitrogen of about the same order as guinea pig and human serum.

The availability of sera of known antibody content has made it possible to obtain quantitative information about the amounts of antibody required for the production of immune reactions *in vivo*. Studies on passive anaphylaxis in the guinea pig have shown that as little as 0.03 mg. of rabbit or guinea pig antiovalbumin nitrogen or of rabbit type III anticarbohydrate nitrogen is required to sensitize an animal so that uniformly fatal anaphylactic shock occurs on subsequent injection of a given amount of antigen (93, 94). With such minimal sensitizing doses of antibody, a large excess of antigen is required so that the reaction takes place in what would correspond to the inhibition zone in the precipitin reaction. Using known amounts of antibody

over a wide range, Follensby & Hooker were unable to demonstrate passive sensitization with equine antihemocyanin and equine antiovalbumin, although passive sensitization could readily be effected with rabbit antihemocyanin (95). Adoption of the procedure of using known amounts of antibody and antigen, wherever possible for the study of reactions *in vivo,* should result in less difficulty in comparison and evaluation of results in different laboratories. All too frequently discrepancies may be attributed to differences in the potencies of the sera used.

Several papers have appeared which indicate limitations of the quantitative methods for estimation of antigens and antibodies. Bawden & Pirie (96) found that extracts of milled fiber from tomato leaves infected with bushy stunt virus do not precipitate with antiserum to the virus. The virus in these extracts was combined with chromoprotein and formed a nonprecipitating antigen. Removal of the chromoprotein mechanically or by enzymatic digestion restored the precipitating power of the virus.

Chemically altered proteins or protein mixtures also show altered immunological properties. Continuing his earlier studies, Kleczkowski (97) observed that when pairs of proteins are heated, they combine in the initial stages of denaturation to form complexes. If one protein is present in larger amount the complex will not precipitate with antiserum to the minor component although it can still combine with antibody as demonstrated by inhibition tests. This failure to precipitate with antiserum to the minor component could be reversed by peptic digestion. If both proteins are digestible by pepsin the process must be interrupted before digestion is complete. Addition of antiserum to the protein present in larger amount precipitates the entire complex (97). For other factors affecting the use of quantitative methods see (5, 42 to 45).

Antibodies.—Several additional papers on the physical and chemical properties of antibodies have appeared since the last reviews (5, 6) were published. Cooper (98, 99), using electrophoretic separation techniques, found the antibody responsible for positive serological tests for syphilis to be associated with fractions of serum containing beta or gamma globulins. In a similar study (78) in which separations of the gamma globulin into two fractions were made, it was concluded that the Wassermann antibody in syphilitic serum migrated between the beta and gamma globulin fractions although its concentration was too low to be visible as a distinct peak. Ultracentrifugal studies (78)

in whole serum also showed that a portion of the antibody was of higher molecular weight than the gamma globulin. Examination of antibody purified from syphilitic serum by dissociation of washed floccules of Kahn antigen and antibody with 15 per cent salt solution and ether showed that it was associated with two components with sedimentation constants of 7 and 19 Svedbergs, and had a mobility between that of beta and that of gamma globulin. The flocculating and complement fixation titer of purified antibody was increased two to four fold by addition of serum albumin or inactivated whole serum. A thermolabile protein fraction has been isolated from whole serum which inhibits the Kahn and Kline flocculation reactions and which constitutes 0.6 to 1 per cent of the total serum protein (79).

Several attempts have been made to distinguish the antibodies responsible for false positive serological tests for syphilis, such as frequently occur with malaria, infectious mononucleosis, mumps, post vaccinial reactions, etc., as well as in certain apparently healthy individuals (cf. 21), from the antibody associated with syphilis (78, 98, 99, 100). While differences in the electrophoretic patterns of syphilitic and false positive sera have been noted (98, 99, 100), these differences do not in general permit conclusions to be drawn in any given case. No differences attributable to the substances responsible for true and false positive reactions could be detected by assay of fractions separated electrophoretically, by ultracentrifugal studies on whole sera, or by application of the salt dissociation method for the purification of antibody (78). Whatever slight variations were observed appeared to be associated with differences in the potency of the various sera. The large majority of syphilitic sera are of high titer, whereas a very high proportion of false positive reactions is of low titer. Obviously, therefore, any method which in effect lowers the sensitivity of the test will eliminate most of the false positive reactions without affecting the reactions of most of the syphilitic sera. The crux of the problem, however, involves differentiating between syphilitic and false positive sera of equal titer, and before any test is accepted as useful, it should be able clearly to distinguish between such selected sera. Thus far, it has not been satisfactorily demonstrated that any of the procedures advocated (100) satisfies this criterion, and it appears to the reviewer that many of them merely partially destroy or inhibit the antibody, so that the net effect is to eliminate the weak reactions, the majority of which are false positive.

Ultracentrifugal studies have shown the isoagglutinins A and B

in human serum to have a sedimentation constant of 19.8 Svedbergs (101), similar to that found for a portion of the human Wassermann antibody (78). Oudin & Grabar (102) have found that a portion of the egg albumin rabbit anti-egg albumin specific precipitate is soluble in 15 per cent salt solution. Only very small amounts of purified antibody could be obtained, however, since most of the material precipitated on dialysis against physiological saline. Similar results were obtained in attempts to purify isoagglutinins (77). Svensson (103) was able to purify antibody by first adsorbing antigen on charcoal, adding antiserum, washing free from excess protein, and eluting the antibody from the charcoal with 0.1 N acetic acid.

As by-products of the large scale fractionation of human plasma for the preparation of albumin during the war, globulin concentrates rich in isoagglutinins and various antibodies usually present in human serum have become available (104, 105, 106). A gamma globulin concentrate has proven highly successful in the prophylaxis and treatment of measles. Preliminary studies indicate some value in therapy and prevention of scarlet fever (106).

Eggerth (107) studied the effects of ninhydrin on horse and rabbit antibodies and found them to be similar in many respects to those of formaldehyde. In horse antisera, antipneumococcal antibodies and the typhoid O-agglutinins were partially destroyed, whereas the H-agglutinins were unaffected. In rabbit antisera mild ninhydrin treatment increased the activity of the typhoid H-agglutinins without affecting O-agglutinins or antipneumococcal antibodies. Tyler (108) reported that photooxidation of rabbit antisera to sea urchin sperm, pneumococci, or sheep erythrocytes converted the antibodies into a nonprecipitating, nonagglutinating form which could still combine with antigen to inhibit reactions with unaltered antibody. Quantitative agglutinin determinations on photooxidized antipneumococcal sera showed no decrease in antibody nitrogen content; the error, however, is stated to be about 25 per cent. Hemolytic activity of antisera to sheep erythrocytes decreased rapidly on photooxidation although their capacity to inhibit agglutination increased. For other studies on chemically altered antibodies see (5, 6, 109, 110).

The use of adjuvants in immunization to obtain an enhanced antibody response has become increasingly popular. The procedure introduced by Freund & McDermott (111) of incorporating the antigen in a lanolin-like substance (aquaphor or falba), which is then emulsified with a suspension of tubercle bacilli or other acid-fast organisms

in paraffin oil and injected, has been widely used in this country. It has proven effective in stimulating precipitin formation to horse serum (111) and to egg albumin (94) in guinea pigs, and in producing diphtheria antitoxin and typhoid agglutinins in rabbits (112), and antibrain antibodies in monkeys (113). Passive transfer of cutaneous sensitivity has been accomplished with guinea pig antiserum produced in this way (114, 115, 116). Better immunity and antibody response to influenza virus in mice and rabbits have been obtained with these adjuvants than with influenza virus alone (117). Incorporation of staphylococcus toxoid into a vaccine of *Plasmodium lophurae* also produced an enhanced immune response in ducklings (118). Freund *et al.* have also used his technique (111) to immunize ducks and rhesus monkeys to formalinized *P. lophurae* and *knowlesi* respectively and have conferred protection against otherwise fatal doses of malarial parasites (119, 120). Ramon (121) advocates addition of tannin to diphtheria toxoid as an adjuvant and Halbert *et al.* (122) have found that incorporation of toxic materials in an emulsion with mineral oil permits the use of higher concentrations for immunization and gives an enhanced antibody response.

For the detection of small amounts of antibody, a number of workers have adsorbed the antigen on a variety of particles and have carried out agglutination tests for antibody. Collodion (123) and carmine (124) particles and bacteria (*S. marcescens*) (125) have been used for this purpose and have proven of value in the demonstration of antibodies in the sera of patients sensitive to ragweed (123, 126) and to insulin (127), as well as of antibodies to distemper virus in dog serum (128), and of antibodies to tuberculin (129, 130). Lund (131) has developed a method for the titration of traces of syphilitic antibody.

In pneumococcus pneumonia treated with sulfonamides, recovery appears in part to be determined by the capacity of the patient to form type specific antibodies. About two-thirds of the patients so treated were found to develop antibodies (132, 133) as measured by mouse protection tests. For quantitative studies on the amounts of antibody formed as a result of pneumococcus infection see Heidelberger & Anderson (75). Normal rabbit sera have been found to contain natural antibodies capable of fixing complement in the presence of saline extracts of normal rabbit tissues (134). The antigen responsible for this reaction is sedimentable at high speed (134). Specific protection of the chick embryo against infection with *B. dysenteriae* (Flex-

ner) has been effected with antisera of the homologous type (135). Methods for the assay of plague antisera for protective and antitoxic antibody have been described by Jawetz & Meyer (136). The induction of mutations by antibodies has been reported by Emerson (137). Injection of rabbit antisera to rat lymphosarcoma was reported to produce regression of tumors (138). Cold hemagglutinins have been found in the serum of patients with kala-azar (139).

Of considerable interest is the finding of Levine & Gilmore (140) that certain patients with infectious mononucleosis have sera which do not agglutinate sheep erythrocytes but which possess the capacity, if added to sheep red blood cells, of inhibiting their agglutination by sera from other patients containing heterophile agglutinins. These observations were taken to indicate the presence of two different kinds of antibody : one which agglutinates and the other which combines with antigen without causing agglutination.

Similar observations had previously been made with the sera of Rh-negative mothers of erythroblastotic infants. It was observed that only about 50 per cent of such sera contained anti-Rh agglutinins (141). Studies on sera in which Rh agglutinins could not be demonstrated revealed the presence of antibodies which combined with Rh-positive cells without causing agglutination (142, 143). These antibodies have been termed "incomplete" or "blocking" antibodies (142, 143), and have been found to be more stable than the agglutinating or complete Rh antibody (145). Methods for the demonstration of these antibodies have been developed by Wiener and by Diamond et al. (145 to 148). Witebsky et al. (149) have demonstrated the presence of anti-Rh in the breast milk of a woman with an erythroblastotic child, and call attention to the dangers of breast feeding in such instances. Sodium ethyl mercuri thiosalicylate was found to inhibit Rh agglutination without affecting the A or B isoagglutinin system (150). The presence of hemagglutinins for human cells in 66 of 100 guinea pig sera was reported by Ecker et al. (151). For the production of Rh typing sera the selection of guinea pigs whose sera do not contain such agglutinins is advocated.

Several groups of investigators have been engaged in identifying the cells in which antibodies are produced. Considerable evidence has been amassed that lymphocytes form antibodies. Earlier studies of Hudack & McMaster (152) had established that antibody formation took place in lymph nodes, and Harris et al. (153, 154) observed that the antibody titer of extracts of lymphocytes was higher than that of

lymph plasma. Antibodies from lymphocytes were found to pass into the lymph plasma, but lymphocytes did not absorb antibodies from the plasma (153, 154). The difference between the antibody titer of lymphocyte extracts and lymph plasma was greatest at the time when the greatest increase in antibody content of whole lymph occurred (153, 154). Independent evidence in support of this concept has been obtained as a result of studies on the effects of adrenal cortical and pituitary adrenotropic hormones on lymphoid tissue (155 to 158). Injection of these hormones into mice, rats, or rabbits resulted within three to six hours in a lymphopenia and a decrease in the number of lymphocytes in lymphoid tissue together with a concomitant increase in serum protein, chiefly globulin (155, 156, 156a). Injection of either hormone into rabbits previously immunized with sheep erythrocytes produced an increase in titer within six hours and a return to the original level after twenty-four hours (157). Extracts of lymphocytes contained both antibodies (158) and protein with the mobility of gamma globulin; the presence of gamma globulin in lymphocyte extracts was demonstrated immunologically by Kass (159). Injection of either hormone into rabbits immunized with sheep erythrocytes, but with serum titers which had been allowed to decline produced a sharp anamnestic rise in antibody titer within five to ten hours; no such response could be elicited by antigen (158). White and his collaborators interpret these phenomena as indicating release of antibody from lymphocytes during this interval under hormonal influence.

The plasma cells, which are generally considered to be derivatives of lymphocytes, also are believed to play a role in antibody formation. Bjorneboe & Gormsen (160) have shown that intensive immunization of rabbits with polyvalent pneumococcus vaccine results in a massive proliferation of plasma cells. They also report a close correlation between the amount of antibody formed and the proliferation of plasma cells; increase in spleen weight during such immunization is due to plasma cell proliferation.

It should be emphasized that none of the evidence advanced in support of the role of the lymphocyte or of the plasma cell in antibody formation in any way contradicts or weakens the mass of evidence previously accumulated involving the phagocytic cells of the reticuloendothelial system in this process. Indeed, all three kinds of cells may produce antibodies. With respect to both the plasma cells and the lymphocytes, there is as yet no evidence to explain how these nonphagocytic cells take up particulate antigens.

Antigens.—Studies on the immunological specificity of fibrinogen and fibrin have established that heat-coagulated beef fibrinogen, Ca-fibrin, T-fibrin, and heat-coagulated Ca-fibrin all produce precipitins to fibrinogen, indicating no gross changes in antigenicity between fibrinogen and fibrin (161). Neither albumin nor pseudoglobulin appears to be present in either fibrinogen or fibrin (161). Studies on serum fractions by Janeway (162) indicate that albumin and gamma globulin in horse serum are immunologically unrelated, but that cross reactions occur between antisera to these fractions and the alpha and beta globulins; whether this is due to contamination of these latter two fractions has not been ascertained; similar results have been obtained with fractions from bovine serum. The anticomplementary properties of human gamma globulin and their inhibition by albumin have been studied by Davis *et al.* (163); electrophoretically separated human gamma globulin has also been found to give a paretic type of colloidal gold curve and a positive cephalin flocculation test (164). A crystalline globulin which gave a positive Wassermann reaction crystallized spontaneously from the serum of a patient with chronic arthritis (165).

Antisera to hog metakentrin, one of the pituitary hormones, do not react with metakentrins from other species or with other pituitary hormones from hog glands; these antisera may be used to estimate the extent of contamination of other products with metakentrin (166). Neutralizing antibodies, but no precipitins, may be produced to renin, the pressor substance of the renal cortex, by injection of renin into a heterologous species; antirenin is found in the pseudoglobulin fraction (167). Antibodies to the gonadotropic activity of extracts of sheep pituitary have been produced in rabbits; antibodies to sheep serum protein were also formed (168).

Crystalline *d*-ribonuclease from beef pancreas has been found to be antigenic in rabbits (169); when combined with antibody a 10 to 30 per cent decrease in enzymatic activity was observed.

Pangborn (170) reports a phospholipid from heart, cardiolipin, which in the presence of lecithin and cholesterol possesses the property of fixing complement with syphilitic sera; for conflicting data see (171). Maltaner & Maltaner (172) have standardized suspensions of cardiolipin, lecithin, and cholesterol for complement fixation tests for syphilis. Both the Wassermann and Forssman activities of aqueous extracts of tissues have been shown to be sedimentable in the ultra-centrifuge at 27,000 RPM for one hour (173). Heidelberger & Mayer

(174) found that normal human stromata give complement fixation with the sera of patients with relapsing vivax malaria.

Kidd (175) has shown that antibodies to the distinctive antigen obtainable from Brown-Pearce rabbit tumors are capable of suppressing tumor growth when mixed with suspensions of living tumor cells. In many instances regression of tumors occurred in rabbits with high titers of antibody whereas metastases and progressive growth usually was noted in animals which had little or no circulating antibody.

Using a technique similar to that for blood grouping, Snell (176) has been able to demonstrate antigenic differences between the sperm of several different inbred strains of mice.

Several groups of workers have been interested in the blood group substances chiefly because of the suggestion by Witebsky et al. (177, 178) that these specific substances be added to group O blood to neutralize the isoagglutinins prior to its use for inter-group transfusions. Clinical studies have indicated the value of this procedure and purified preparations of A and B substances for this purpose are available commercially. Aubert, Boorman & Dodd (179) reported an increase in isoagglutinin titer following injection of A individuals with group B plasma and of B individuals with group A plasma, and Witebsky et al. (180) have demonstrated that purified blood group A and B substances are antigenic in humans who do not possess the corresponding isoagglutinogen and that potent antisera for use in blood grouping may be obtained by injection of these substances. Such sera can be used for quantitative precipitin studies (77). Wiener et al. (181) have also used autoclaved saliva from secretors of blood groups A and B for the preparation of typing sera. Other studies on isoimmunization by transfusion, with the production of agglutinins for A, B, Rh and for isoantibodies to A_1 have been made by various workers (182, 183, 184).

Elegant methods for the purification of the blood group A substance from hog gastric mucin have been developed by Morgan & King (185). In one method the mucin is extracted with 90 per cent phenol and the A substance precipitated from the clarified phenol extract at a concentration of 10 per cent alcohol; the second procedure involves extraction of the mucin with saline saturated with carbon dioxide, clarification in a Sharples centrifuge, and precipitation of the A substance with sodium sulfate between 27 and 30 per cent by weight. Preparations obtained by both methods were equal in potency as measured by their capacity to inhibit isoagglutination and by sheep

cell hemolysis. Preparations of A substance contained from 5.5 to 6.1 per cent nitrogen, 9.2 to 11.5 per cent acetyl, and 27 per cent glucosamine. About 20 per cent of the material consisted of amino acids (cf. 186). On heating, a decrease in capacity to inhibit isoagglutination and an increase in hemolysis-inhibiting power was noted (185, 189). Morgan & Van Heyningen (187) found that pseudomucinous ovarian cyst fluids from secretors of blood groups A, B, and AB were rich in blood group substances. The substance from human cyst fluid differed from the hog A substance in that it was insoluble in 90 per cent phenol. Purified preparations of human A substance contained 6 per cent nitrogen and 10 per cent acetyl (189). Both human and hog A substances may be made to function as antigens in the rabbit if combined with the protein from the Shiga dysentery bacillus (23, 190), a procedure previously used to form antibodies in rabbits to agar, gum acacia, and other plant gums (190). With such antisera, immunological differences between the human and hog A substances have been reported (187). Jorpes & Thaning (188) also studied the blood group A substance from human urine and from the stomachs of cattle. Oliver-Gonzalez & Torregrosa (191) found a variety of parasites to contain a substance or substances related to the human A and B substances and to the Forssman antigen, and also reported high titers of anti-A in malaria patients of groups O and B who had suffered repeated attacks. Pernicious anemia did not appear to affect the incidence of group specific substances in the gastric juice (192). Boyd & Warshaver (193) reported four cases in which injection of group B cells into rabbits resulted in the appearance of agglutinins for A cells as well as for B cells; in two of these instances anti-A and anti-O precipitins were noted. The demonstration by Witebsky & Mohn (194) that Rh substances occur in amniotic fluids should materially facilitate attempts at isolation and characterization of these substances. The Rh factor in the amniotic fluid appears to be of fetal rather than maternal origin. As with the A and B factors, there appear to be Rh secretors and nonsecretors.

Studies on bacterial antigens in which quantitative methods were employed have been cited above. Bovarnick (195) has studied conditions for the production by *B. subtilis* of the *d*-glutamyl polypeptide previously isolated from *B. anthracis* and *B. mesentericus* by Ivanovics. By disintegrating live cells of *Brucella abortus* and *B. suis*, Huddleson obtained soluble fractions which could actively immunize guinea pigs against brucella infections (196).

Kauffmann (197) demonstrated the presence of a thermolabile antigen (L) in strains of *E. coli* which inhibits the agglutination by its homologous antibody of the somatic O antigen in living cultures. Kauffmann has also been able to classify a large number of coli strains into 58 groups based on their O antigens; most of these contain L antigen and are non-agglutinable by O antiserum unless heated. L antibodies in sera may be demonstrated with living cultures and appear to be associated with protection. The L antigens appear to be toxic for mice since non-agglutinable strains are more toxic than agglutinable strains. A number of coli strains were found to contain antigens related to the O antigens of salmonella and to the Flexner group of dysentery bacilli. Kauffmann (197) also described several weakly agglutinable strains of *E. coli* whose agglutinability is unaffected by heating. These are referred to as "A forms." Subsequent study by Knipschildt (198) showed that these A forms contain a thermostabile capsular antigen (A) which also is capable of inhibiting O-agglutination; thirteen serologically distinct A antigens have been found; two A strains cross react with antisera to type 23 pneumococcus.

A classification of *Shigella paradysenteriae* (Flexner) based on the identification of fourteen primary types has been effected by Weil *et al.* (199, 200); several types with two primary antigens have been found. From passive protection tests with typing sera in the chick embryo, it appears that the antigens used in classification are those associated with protection (135). This observation should materially facilitate selection of strains in various localities for active immunization. Non-agglutinable strains of dysentery bacilli were also noted; the factor responsible for inhibition of agglutination is destroyed by heat (199, 200); other studies on the classification of dysentery bacilli have been made by Kauffmann (201). Goebel *et al.* (68, 202) obtained a lipocarbohydrate protein complex from *Sh. dysenteriae* (Flexner) type V which is antigenic and highly toxic. Serum from humans after immunization with this antigen show a marked rise in mouse protective antibodies.

The specific carbohydrate of type I meningococci has been prepared from cultures grown on synthetic media (203). Electrophoretic separation, precipitation with safranin or protamine, or fractionation with methyl and ethyl alcohol has been used in purification of the type I polysaccharide (76); these products when tested with antimeningococcal sera show only a very narrow zone in which supernatants contain both polysaccharide and antibody, indicating the absence of most

of the contaminating substance previously found in type I preparations
(204, 205). Production of type-specific active immunity in mice as
well as the development of type-specific horse antibodies protective
for mice was obtained by injection of broth filtrates of type I men-
ingococci (206); whether the type-specific carbohydrate is responsible
has not been established. Menzel & Rake (207) isolated a type-specific
substance from broth autolysates of type II meningococcus cultures,
called Kappa. Kappa is associated with type-specific protection, and
may be liberated from intact cells by proteolytic enzymes. Boivin-
type antigens have been isolated from meningococci and gonococci by
Boor & Miller (208), and Stokinger *et al.* (209) have carried out an
analysis of the constituents of gonococci and have isolated a toxic and
antigenic protein from *N. gonorrhoeae* which fixes complement with
rabbit antisera and with sera of patients with gonococcal infections.
Phair, Smith & Root (210) have advocated the use of chicken sera
for typing meningococci.

Goebel *et al.* (211) have isolated and studied the group-specific
"C" carbohydrate and the heterophile "F" antigen of pneumococcus.
Both products are very similar in chemical composition except that
the F substance contains about 6 per cent of a fatty acid which is lib-
erated on hydrolysis. The F polysaccharide is antigenic in rabbits
and gives rise to precipitins and sheep cell hemolysins, while the C
substance is not antigenic in the rabbit (212). From chemical and
immunological studies, including quantitative precipitin determina-
tions, Goebel *et al.* (211, 212) concluded that the C substances com-
bined with lipid constitutes the heterophile antigen of pneumococcus.

Hehre & Sugg (213) described the synthesis from sucrose, by cell
free extracts of *Leuconstoc mesenteroides,* of polysaccharides which
show cross reactions with antisera to types 2, 12, and 20 pneumococci
as well as with homologous antiserum. Similar products with sero-
logical reactivity for other types were also obtained with enzymes from
Rhizobium strains (214) and from *Streptococcus salivarius* (215).

Reference is made to earlier work by W. T. J. Morgan, Freeman,
H. R. Morgan, and others on the Boivin-type antigens of the sal-
monella-typhoid group of organisms (5, 6, 16, 17). More recently
Freeman (216) has prepared the antigenic complex of *S. typhimurium*
by ethanol and ammonium sulfate fractionation in a form which he
believes represents a chemical entity. It is broken down into four
components on acetic acid hydrolysis, and is composed of 69 per cent
of a specific polysaccharide containing *d*-glucose, *d*-mannose, and

d-galactose, 16 per cent of a conjugated protein, 3 to 4 per cent lipid, and 8 per cent of an alcohol-soluble acetylated polysaccharide.

Purified somatic antigens from typhoid and paratyphoid A and B organisms produce less local and general reaction in humans than do the standard TAB vaccine, and are more effective in inducing the formation of protective antibody (217). Linton *et al.* (218) have studied the electrophoretic properties of concentrated broth cultures containing the various typhoid antigens.

The property shown by the Boivin-type O antigens (endotoxins) from a variety of Gram negative bacteria of producing tumor hemorrhage in animals with transplantable tumors has been extensively studied (219 to 226). These endotoxins are also capable of interrupting pregnancy in mice and rabbits at certain stages due to a hemorrhagic action on the placenta and decidual tissues presumably analogous to that on tumors (222). Endotoxins prepared by a variety of methods (223, 224) were toxic, hemorrhage-producing, and antigenic; sulfanilamide inhibited both the toxicity and hemorrhagic action on tumors. Mice immunized to the endotoxin of *S. typhimurium* are protected to a limited extent against both tumor-hemorrhage and the toxic effects of heterologous endotoxins from *Sh. paradysenteriae* (Flexner), *Rhodospirillum rubrum,* and against moccasin venom (223, 224, 225), which also produces hemorrhage in tumors (226); protection afforded against the homologous O antigen is no greater than against heterologous endotoxins (225). Much evidence was presented indicating that the same substance is responsible for toxicity and for hemorrhage induction in tumors (219 to 226). The hemorrhage-producing fraction from *S. marcescens* has also been extensively studied by Shear and co-workers (227, 228). The material was purified from culture filtrates on synthetic medium by alcohol precipitation and shaking with chloroform. The active fraction was contained in the chloroform layer. Shear *et al.* consider their product to be a lipopolysaccharide, since they were unable to demonstrate the presence of protein or polypeptide. The bulk of the nitrogen was not identified and it is still possible that they were dealing with a Boivin-type antigen. The active fraction from *S. marcescens* could not be shown to be definitely anaphylactogenic (229).

The preparation and characterization of the type-specific M proteins, the protective antigens of the hemolytic streptococcus, have been considered in detail (5, 18). Recent studies by Lancefield and coworkers (230 to 233) have shown that proteolytic enzymes such as

trypsin, chymotrypsin, and pepsin destroy the type-specific M substance even in the living cell. Injection of enzyme-treated cultures into rabbits yields no antibodies to the M antigen, but provides specific antisera to the T substances, the antigens of hemolytic streptococcus which are responsible for type-specific agglutination but are unrelated to type-specific protection. With these sera certain strains of group A streptococci were shown to be lacking in T antigen; other strains containing T antigen do not agglutinate with homologous anti-T sera because agglutination is inhibited by large amounts of M substance. Strains of type 10 and 12 hemolytic streptococci could be shown to contain identical M but different T antigens. Closely related T antigens were shown to be responsible for cross-reactions observed among a number of different types. Group A streptococci have also been shown to produce an extracellular proteolytic enzyme which specifically attacks all the type-specific M antigens except that of type 28 (234). Loss of group-specific carbohydrate in a type 27 hemolytic streptococcus was shown to result from rapid passage through mice (235).

Seibert's studies on tuberculin proteins have been reviewed recently (5, 19). McCarter & Watson (236) have found the intracutaneous activities of tuberculins for human beings to correlate with their antigenicity regardless of whether the proteins were derived from human or avian tubercle bacilli. With antigenic preparations as many as 100 per cent of the individuals of a rural population with a low incidence of tuberculosis may show positive reactions after six hours. Antigenicity of the tuberculin protein is destroyed when the molecule is degraded by heat to a molecular weight of about 6,000. Trypsin and chymotrypsin almost completely destroy the activity of tuberculins; peptic digestion at pH 2 and 3.5 results in a product with one-tenth the potency (236, 237).

Smolens & Mudd (238) have prepared an antigenic, nontoxic substance from *H. pertussis,* phase I, which completely absorbs agglutinins from pertussis antisera, and has been used in skin tests for susceptibility to whooping cough.

Several workers (239, 240, 241) have described the preparation of tetanus toxin and toxoid. A new quantitative method (242) for the estimation of formaldehyde in biological mixtures should prove useful in studies on toxoid formation.

The early diagnosis of typhus has been greatly facilitated by the introduction of a complement fixation test for circulating Rickettsial

antigen (243) to replace the Weil-Felix reaction. Antigens from egg-grown Rickettsia have been studied (244), as have also the serological relationships between Rickettsia and *B. Proteus* (245, 246). Immunological studies on the psittacosis-lymphogranuloma group of viruses have been carried out by Hilleman (247). An immunologically active polysaccharide, containing galacturonic acid, glucose, and an unidentified sugar constituent, was isolated from filtrates of *Coccidioides imitis,* the causative agent of coccidioidomycosis (248). The polysaccharide precipitates with antisera and gives a positive skin reaction in sensitive individuals. Boiled aqueous-extracts of *Trichinella spiralis* larvae have been advocated for the serodiagnosis of trichinosis by complement fixation (249). A serologically active polysaccharide has been obtained by Melcher & Campbell (250) from rabbits infected with trichinella; another immunologically active polysaccharide, previously isolated by Campbell (251, 252) from ascaris, has been shown to consist chiefly of glycogen (253). Anderson, Goodpasture & DeMonbreun (254) have demonstrated by immunological methods an etiological relationship between granuloma inguinale and Donovania granulomatis and have obtained from these Donovan bodies, grown in egg yolk, a mucoid material giving specific precipitation and complement fixation with sera of patients with this disease.

The chemistry and immunochemistry of the allergenic substances responsible for cottonseed hypersensitivity have been extensively studied by Spies, Coulson and co-workers (255 to 259). A protein-polysaccharide complex, CS-1, was isolated from cottonseed by a procedure involving aqueous extraction, heat, fractional precipitation with alcohol, and removal of impurities with basic lead acetate; a similar product has been obtained from castor beans (256). This fraction contains the principal allergenic constituents and could be further purified and freed from carbohydrate by precipitation with picric acid, chromatography, and adsorption and elution from activated aluminium oxide. The picric acid could be removed with alcohol to yield a purified proteose-like allergen, CS-13, which is strongly anaphylactogenic in guinea pigs. Picrates of electrophoretically separated fractions of CS-1 were also found to be anaphylactogenic; the anodic fraction was more effective in inducing sensitization than was the cathodic fraction although the specificity of both was identical. The capacity of these two fractions to sensitize and elicit shock in guinea pigs was sharply reduced by boiling in $0.1\ N$ hydrochloric acid

as was their ability to neutralize skin sensitizing antibody although
their capacity to elicit skin reactions in cottonseed-sensitive individuals
was found to be unaffected. Cottonseed globulin was also shown to be
contaminated with CS-1. Several distinct allergens have been demon-
strated in cottonseed (26, 27, 259). Abramson, Moore & Gettner
(260) have obtained a protein or polypeptide-like substance with a
molecular weight of about 5,000 by electrophoretic separation of rag-
weed extracts.

Chemically altered antigens.—The introduction of known chem-
ical substituents into proteins has continued in use as a means of ob-
taining antisera with a specificity directed toward well-defined chem-
ical configurations. Singer prepared antisera to diazotized arsanilic
acid coupled to ox globulin and found that these antisera would pro-
tect mice against otherwise lethal doses of resorcinol derivatives of
arsanilic acid containing at least two or three arsonic acid groups, but
not against compounds with one group such as p-aminophenylarsonic
acid or phenol p-azophenylarsonic acid (261). The contraction of
sensitized smooth muscle strips induced by simple substances with
several haptenic groups could be inhibited by compounds containing
but a single haptenic group (262). Fell and co-workers (263, 264)
prepared histamine-protein antigens by coupling diazotized p-amino-
benzoyl histidine or the isocyanate of histamine to proteins. Guinea
pigs immunized with these histamine protein conjugates were found
to be protected against anaphylactic shock. In the writer's laboratory,
immunization with histamine azoprotein, however, was found not to
decrease the susceptibility of guinea pigs to intravenously injected
histamine; the protective effect against anaphylactic shock could be
shown to be nonspecific since similar results could be obtained using
normal serum instead of histamine azoprotein (265). Anaphylactic
shock has been produced in guinea pigs sensitized with sulfonamide
azoproteins by intravenous injection of sulfonamides coupled with
heterologous protein (266).

Pressman, Campbell & Pauling (267) have coupled erythrocytes
with diazo compounds and with other proteins through azo linkages
without marked destruction of the erythrocytes and have been able to
demonstrate agglutination of such erythrocytes by antisera homol-
ogous to the introduced group. Merthiolate, a substituted benzoic
acid, in concentrations usually employed for preservation of serum,
has been found to interfere with precipitin reactions involving anti-
sera prepared with azoproteins containing benzoic and arsonic acid

groups. In some instances inhibition of precipitation occurred in the presence of merthiolate and in others increased precipitation was noted (268).

Complement.—Recent studies (269) on titration of each of the four components of complement using the principles enunciated by Hegedus & Greiner (270) have established that C'3 and C'2 are the components present in lowest titer in guinea pig and human serum respectively. Two groups of workers now appear to agree that, in immune hemolysis, each component of human complement may be replaced by the corresponding component of guinea pig complement and vice versa (269, 271). Details of procedure and of precautions in the titration of the individual components of complement are given by Bier *et al.* (269). The bactericidal action of human complement and antibody on the cholera vibrio has been found to be destroyed by inactivation of any one of the four components, and may be restored by addition of the missing component (272, 273). Ecker and co-workers consider the bactericidal and hemolytic properties to be dependent on the same components (272, 273). Individual components showed no bactericidal action. For earlier studies and references see (5, 12).

Immune reactions.—Several workers have advanced theories of the mechanism of the precipitin reaction (8, 274, 275). Kendall (274) modified the theory previously developed by Heidelberger & Kendall (276) and was able to account quantitatively for the course both of the precipitin and the toxin-antitoxin types of reaction, on the assumption that antibodies showing the former type of behavior have two similar reactive groups per molecule and those showing the latter type of behavior have two different kinds of reactive groups per molecule. Although based on different assumptions the equations derived by Pauling (8) are of the general form as those originally proposed by Heidelberger & Kendall (276). Hershey (275) has developed a theory from other considerations. From his analysis, contrary to Pauling (8, 81 to 91), an aggregate of bivalent antigen and bivalent antibody would not be expected to precipitate.

From quantitative studies on the inhibition and precipitation reactions of simple substances containing one or more hapten groups per molecule, Pauling and his co-workers (81 to 91) have advanced evidence in support of the framework theory of antigen-antibody reactions and of the bivalence of antibody. Pauling *et al.* (84) express the effectiveness of a hapten in inhibiting precipitation of a given precipitating system in terms of a "bond strength constant" obtained from

the initial slope of a plot of the amount of antibody precipitated against the molal concentration of hapten. The validity of the evidence in support of the framework theory and of the calculations of antibody valence and, by implication, the value of the entire approach of the Pauling group (81 to 91) to the study of antigen-antibody reactions using simple substances have been sharply challenged by Boyd & Behnke (277), who found that one of the haptens VII (XI) used by Pauling was aggregated in solution. The formula weight of the hapten was 1,122, but from diffusion measurements, a particle size of 12,500 was calculated, indicating about 10 to 12 molecules per particle. Such aggregated particles would certainly not be expected to have the valence assigned to them from their formula weights. While it is not known exactly how many of the haptens used by Pauling *et al.* (81 to 91) are aggregated in solution, it is possible that a considerable number, if not all of those thought to contain more than one hapten group, may prove to be aggregates, and precipitation may actually be an attribute of a multivalent hapten and inhibition a property of a monovalent or perhaps divalent (cf. 275) hapten. It is especially disappointing to note that, as yet (one and one-half years later) no answer to this challenge has been made by the Pauling group. While the writer is a strong supporter of the framework theory, he nevertheless must subscribe to these criticisms. The findings of Bukantz & Abernethy (278) that sulfonamides coupled to resorcinol precipitate and fix complement with normal and antipneumococcal rabbit sera and that solutions of the sulfonamide drugs inhibit this precipitation also indicate a potential source of error in studies with chemically altered antigens and simple haptens. The possibility that such unrelated reactions may occur should be tested carefully in all studies involving chemically altered antigens and haptens.

Grabar & Oudin (279) have carried out studies on the reaction of egg albumin and rabbit anti-egg albumin in a medium containing 20 per cent alcohol. Precipitation under these conditions does not occur until antigen first appears in excess; with a large excess of antigen, inhibition of precipitation is not as great as in the absence of alcohol. Boyd & Purnell (280) have considered the differences between the two kinds of optimal proportions flocculation ratios.

Rothen & Landsteiner (281) have found that on metal surfaces films of chicken ovalbumin, horse serum albumins and globulins still combine specifically with antibodies. Monolayers of purified antipneumococcal antibodies were also found to combine with specific

polysaccharides. More recently Rothen (282) has reported that the amount of antibody bound by films of bovine albumin is increased if more than one layer of bovine albumin is first deposited on the slide; no such effect was observed in the binding of homologous antibody by monolayers of egg albumin placed upon one another. Combination of bovine albumin films with antibody occurs even when six to eight layers of stearic acid or of egg albumin are deposited on the slide, presumably over the bovine albumin layers. Results on the binding of anti-egg albumin by egg albumin films after coating with stearic acid appear equivocal. Rothen interprets the bovine albumin data to indicate "that no direct contact is necessary between a film of antigen and the corresponding antibodies to demonstrate a specific interaction" and further suggests that "an interaction might even occur through a thin biological membrane." There is little reason to believe that Rothen's observations are of general significance for antigen-antibody reactions. Indeed, the differences in the behavior of bovine albumin and egg albumin and their homologous antibodies would suggest that other factors apart from the combination of antigen and antibody are responsible. For example, the results may be a function of the ease with which bovine albumin films resume the globular form as contrasted with egg albumin films.

Stats (283, 284) has noted that cold-hemagglutinated erythrocytes hemolyze readily on shaking. Martin has introduced a serum dilution method using minimal amounts of antigen in titrating antibodies in a pure antigen-antibody system (285).

The role of histamine in immune reactions *in vivo,* such as anaphylaxis, has been questioned by Jadassohn *et al.* (286) who tested the relative potency of a group of histamine inhibitors in preventing the contraction of uterine muscle by histamine and of sensitized uterine strips by antigen. Since the order in which the substances used inhibited histamine activity and the anaphylactic contractions was not the same, these workers concluded that histamine is not involved in anaphylaxis; for evidence in favor of the histamine theory see (29, 30).

Two kinds of antibody have been shown to be present in the serum of treated hay fever patients and of patients with other hypersensitivities (127, 287). One of these is relatively thermolabile and can passively sensitize normal human skin; the other is thermostable and combines with antigen to prevent the antigen from reacting with the skin sensitizing antibody. Normal humans produce only the thermostable antibody in response to parenteral injections of ragweed ex-

tracts. Weil & Reddin (288) have demonstrated the presence of both types of antibody to ragweed in cattle. Other studies are reviewed (26, 27, 114, 115, 116, 289).

The findings of Landsteiner & Chase (114, 115) that specific hypersensitiveness of the "delayed type" may be transferred to normal guinea pigs by means of cells from exudates of sensitized guinea pigs is of outstanding interest. This work has been extended by Chase (290), who has for the first time effected passive transfer of the tuberculin reaction with similar cell exudates.

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ORGANIC INSECTICIDES

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INTRODUCTION

The chemical control of injurious insects is becoming an increasingly important branch of applied organic chemistry, since the inorganic poisons are steadily giving way to organic substances, some of plant origin and others purely synthetic. This change is occurring in part because the public dislikes contamination of foodstuffs with materials containing arsenic, fluorine, or other elements toxic to mammals. An even more compelling reason is that the newer insecticides are much more effective against the various pests. The purpose of this article is to discuss briefly the advances made during the last few years with respect to the chemical and physical properties, the origin, mode of preparation, and the uses of the more important organic insecticides. Also, theories regarding modes of toxic action and factors affecting this action will be considered. Unfortunately, European research was seriously interrupted by the war and much that has been done is not available yet. Wartime restrictions on publication of certain information in the allied countries withhold other valuable data.

INSECTICIDES OF PLANT ORIGIN

Nicotine continues to attract much interest, especially because it was relatively plentiful during most of the war period. However, its usefulness is still confined in general to contact or possibly fumigant action against small or soft-bodied insects and stomach action against a limited number of larger forms, mostly Lepidoptera (1). The latter use has led to search for compounds stable enough to last over a considerable period under normal exposure to outdoor conditions. Since nicotine is an alkaloid, β-pyridyl-α-N-methyl pyrrolidine [$K_{b_1} = 1 \times 10^{-6}$, $K_{b_2} = 1 \times 10^{-11}$ approx. (2)], it forms salts with any acid and double salts with many metals and acids. Tests with thirty-one such materials against young codling-moth larvae (3) showed the water-soluble compounds, e.g., laurate, oleate, caseinate, to give poor control both before and after sprinkling (to stimulate

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weathering). The less soluble compounds such as the silicotungstate and Reineckate were effective before but not after sprinkling. The two most effective materials were the bentonite complex and the double metallic salt with cuprous cyanide. Other double salts with copper picrate and with zinc, cobalt, cadmium, or nickel benzoyl benzoate were relatively satisfactory before but not after sprinkling. Doubtless this same advantage of the slightly soluble compounds of nicotine holds with any other chewing insect but various species differ enormously in their susceptibility. Very great differences exist in the effectiveness of various complex metallic salts of nicotine as stomach poisons but certain of them, for example, the cuprous mononicotine cyanide and the cuprous dinicotine thiocyanate, compare favorably with standard materials for control of a variety of Lepidoptera and Coleoptera (4).

On the other hand, the contact action of nicotine is most strongly manifested in the soaps such as the laurate, oleate, or naphthenate (5). Equally high toxicity is shown toward aphids by free nicotine plus the ordinary soluble soaps of the same fatty acids. The fixed nicotines, e.g., the silicotungstate or Reineckate, are useless as contact insecticides.

A number of alkaloids containing two heterocyclic rings occur in the *Nicotiana* and *Anabasis* genera, and related compounds have been obtained synthetically. The commercial sources of nicotine are limited as yet to *N. tabacum* and *N. rustica* and it is entirely a by-product of the tobacco industry. Insecticidal uses of nicotine amount in the United States alone to over five hundred tons per year and the total cost to agriculture runs into millions of dollars. Accordingly, there is lively interest in related compounds. Among these, neonicotine (β-pyridyl-α-piperidine) was first synthesized and shortly thereafter discovered (6) in the *laevo* form as a constituent of the plant *Anabasis aphylla* which grows in Russia, North Africa, and elsewhere. The Russians have greatly developed the use of this material which they call anabasine. It resembles nicotine closely but, being somewhat less volatile, is less effective as a fumigant but more persistent as a contact or stomach poison. Another source is the so-called tobacco tree, *Nicotiana glauca*, which occurs in the southwestern states (7). A very large literature has arisen about this substance and its uses which is discussed and listed in two publications of the United States Department of Agriculture (8, 9).

The compound nornicotine (β-pyridyl-α-pyrrolidine) occurs in

several species of *Nicotiana* and in the Australian plant *Duboisia hopwoodii*. It comprises about 95 per cent of the alkaloidal content of *N. silvestris*, which is a commercial tobacco of the United States and a similar proportion (10) in a certain Maryland strain of ordinary tobacco, *N. tabacum*. Commercial nicotine preparations sometimes contain several per cent nornicotine (11). Being less volatile and less easily oxidized than nicotine, it is a better contact poison (12) but less desirable as a fumigant. A comprehensive review on nornicotine has been given by Roark (9).

A point of great interest in connection with the structural basis of toxic action is that neither anabasine nor nornicotine contains the $=NCH_3$ group which occurs in nicotine and once was thought to be important. It is clear that the β-pyridyl group is essential for high toxicity (13). The evidence regarding a connection between optical activity and toxicity to insects is not definite, for while *l*-nicotine is more toxic than the *dl* form which in turn excels the *d* form, all three isomers have approximately equal toxicity in the case of nornicotine (14). Comparisons do not appear to have been made of *l*, *dl*, and *d*-neonicotine.

As noted earlier, differences in the amount and kind of alkaloid have been found in certain species of the tobacco plant. Over a dozen alkaloids are listed in a recent review (15). If a plant having a sufficiently high content of insecticidal alkaloid could be bred there is a possibility of divorcing industrial nicotine production from the tobacco industry (16). By selective breeding three promising strains have been secured: a hybrid of *N. rustica* for nicotine, a cross of *N. tabacum* and *N. tomentosa* for nornicotine, and one of *N. tabacum* and *N. glauca* for anabasine (17). It appears to be generally true that in these plants having a high alkaloidal content nicotine is the chief alkaloidal constituent.

Considerable light has been thrown recently on the sites of alkaloid synthesis in certain species of *Nicotiana*. It was formerly considered that alkaloids are formed at or near where they are found and this theory was taken for granted by Schmuck *et al.* (18) and other Russian workers who argued that the occurrence of more anabasine than nicotine in grafts of *N. tabacum* on *N. glauca* roots was an example of a root altering the biochemical processes normal to a scion. The careful studies of Dawson (19, 20, 21) have shown that among the species examined nicotine is formed only in the root. Nornicotine is formed from nicotine in the leaves of *N. glutinosa* and of certain strains of *N.*

glauca and *N. tabacum* and, lastly, anabasine is formed in both the root and leaves of *N. glauca*. Proof for these conclusions was obtained from reciprocal grafts of the *Nicotiana* species and the common tomato which normally contains no alkaloid, and from the hybrid *N. tabacum* × *N. glauca*. For details the original articles must be consulted. Aside from immediate application of this work to *Nicotiana* breeding, it suggests that similar experiments should be tried with other plants bearing useful products, for instance, rotenone and related compounds.

Interest in pyrethrum has continued at a high level, since the spectacular performance of this insecticidal material, especially against Diptera, made it the one possibility for control of mosquito-borne diseases when the war took the forces of this country to tropical regions. Ever since the active ingredients, pyrethrins I and II, were shown by Staudinger & Ruzicka (22) to be esters, there has been uncertainty in the minds of numerous investigators regarding the structure of pyrethrolone, the alcohol common to both esters. It consists of a five-member carbon ring with a side chain and was assigned the formula (23)

$$OC-CH(OH)-CH_2C(CH_3)=CCH_2CH=C=CHCH_3,$$
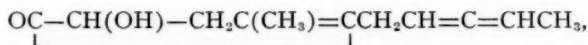
i.e., the side chain was thought to be a 2,3-pentadienyl group. This "cumulated" double bond system had not previously been found in nature and its existence in pyrethrolone has been disputed on the basis of light-absorption data which indicates the more common conjugated double bonds (24, 25). The disagreement apparently has been removed by the discovery (26) that what has been called pyrethrolone actually is two compounds each existing in an optically active and a racemic form. The compounds are

$$OCCH(OH)CH_2C(CH_3)=CCH_2CH=CHCH=CH_2$$
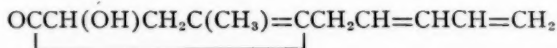
for which the name pyrethrolone is retained, and

$$OCCH(OH)CH_2C(CH_3)=CCH_2CH=CHCH_3$$
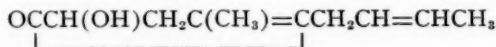
which has been named cinerolone after its plant source, *Chrysanthemum cinerariaefolium*. Cinerolone is more stable chemically than pyrethrolone but no tests have been reported on the insecticidal effects or persistence of a pyrethrin containing exclusively one or the other

alcohol. There thus appears to be not two but four pyrethrins corresponding to the four combinations of two acids and two alcohols.

Comparison of the mortality-dosage curves for the fly, *Musca vicina,* obtained with preparations from *Chrysanthemum roseum* and *C. cinerariaefolium,* indicated much higher mortality with the latter (27). Since dosage was calculated from results of chemical analysis (method unfortunately not stated), these authors postulated a third toxic substance present in *C. cinerariaefolium,* but not responding to the chemical test. This possibly important point has been disputed by Gillam & West (28) who could find no spectral absorption bands attributable to another toxic substance. It has been suggested (29) that an ester with some alcohol other than pyrethrolone might be present in the *C. cinerariaefolium* product. Previously it had been shown that the esters of monocarboxylic chrysanthemum acid with lauryl, myristyl, and cetyl alcohol are highly toxic to aphids but not to the American roach (30). Since esters of the opposite kind, e.g., palmitic and linoleic acids with pyrethrolone (31), and presumably with cinerolone also occur, it is obvious that a complex mixture of substances of various toxicities is obtainable from pyrethrum.

The problem of synthesizing one or more of the pyrethrins has interested numerous chemists since a start was made by Staudinger, Muntwyler & Seibt (32) who succeeded in preparing an optically inactive isomer of the monocarboxylic chrysanthemum acid which gave only a moderately insecticidal ester with pyrethrolone. Recently, as a result of interest in the dienes as starting points for synthetic rubber, the compound 2,5-dimethyl-2,4-hexadiene. $H_3CC(CH_3)=CHCH=C(CH_3)CH_3$, has become available. When this is treated with ethyl diazo acetate, $N_2CHCOOC_2H_5$, and the product hydrolyzed, the acid

$$(CH_3)_2C - CHCH=C(CH_3)_2$$
$$\diagdown\diagup$$
$$HCCOOH$$
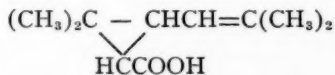
is formed. The *d-trans* isomer is identical with the monocarboxylic chrysanthemum acid of pyrethrin I (33). With the formulas for the alcohols pyrethrolone and cinerolone apparently at last established, and one of the acids synthesized, the total synthesis of pyrethrin I merits serious consideration. The pyrethrins are undoubtedly the most expensive insecticides used, costing nearly twenty-five dollars per pound or roughly ten times as much as nicotine. Hence even a very expensive synthetic process could be operated at a profit.

The uses of pyrethrum preparations have increased greatly during the last few years. The control of disease-bearing insects such as mosquitoes by aerosols containing pyrethrum extracts has been widely publicized and will be discussed later in connection with various improvements in the application of insecticides. Before the outbreak of war pyrethrum products were finding increased use against agricultural pests. This was due in part to the greatly improved raw material from Kenya Colony (34) and to the more satisfactory marketing arrangements with Kenya Colony than with Japanese producers. A limiting factor in large scale use has been the deterioration during storage of either intact or ground flowers and especially of concentrated extracts. West (35) adduced evidence that the alteration is in the unsaturated side chain of the alcoholic constituent which is in agreement with the experimental finding that saturation of the alcohol side chain leads to a marked drop in insecticidal effects (36). Various antioxidants have been found useful in prolonging the activity of pyrethrum preparations, e.g., hydroquinone and pyrogallol (37), and pyrocatechol (38) in the case of concentrated extracts. Isopropyl and diisopropyl cresols were used (0.25 per cent by weight) as antioxidants in the louse powder developed for the armed forces, which contained only 0.2 per cent pyrethrins (39).

Frequently, it has been noted that certain individuals show a sensitivity to pyrethrum. In most cases this becomes evident only after prolonged exposure. The usual symptoms are dermatitis, itching, swelling of the eyelids, and, less frequently, faintness and shivering. The responsible constituent is not known definitely but, according to the results of Martin & Hester (40), it is not the pyrethrins. Using himself as subject, Martin produced intense irritation by application of a volatile oil obtained from the flowers by steam distillation and from a colorless petroleum ether extract of flowers mixed with charcoal. Concentrated pyrethrin extract or pollen were devoid of effect.

For an extended account of recent developments regarding pyrethrum, especially new uses and methods of application, the reader is referred to *Pyrethrum Flowers Supplement 1936–1945* by Gnadinger (41).

The lively interest on the part of many workers in the structure and reactions of the various insecticidal compounds present in the roots of *Derris, Lonchocarpus, Tephrosia, Millettia,* and certain other plant genera has resulted in the accumulation of much information

on these substances. There is general agreement (42) that rotenone is represented by the structure:
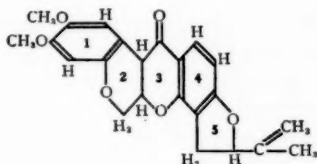
Deguelin has the same empirical formula but contains the following structure in place of the dihydrofuran ring (number 5) and the isopropenyl side chain:
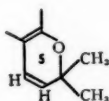
In elliptone this part of the structure is simply a furan ring (43).

If in each of these compounds the upper hydrogen in ring 4 is substituted by a hydroxyl radical, sumatrol, toxicarol, and malaccol are formed which are, respectively, the hydroxy derivatives of rotenone, deguelin, and elliptone. These six compounds plus tephrosin, which has a hydroxyl group on the upper carbon shared by rings 2 and 3, account for most but not all of the toxic constituents of this group of plants.

It will be obvious from the formulas that all these compounds may exist in optically active forms. There is much danger of racemization during separation but the present indications are that these compounds occur in the plants in the *l* forms. The few comparisons of toxicity, made so far, indicate that for each substance the *l* form is considerably more toxic than the racemic mixture (44).

In contrast to the pyrethrins, removal of the double bond in the side chain of rotenone has only a slight effect on toxicity. Saturation of the double bond which is located in ring 5 in deguelin greatly increases the toxicity to house flies (44). These observations are of

practical interest for the hydrogenated compounds are much more
stable to atmospheric exposure and hence may have more persistent
insecticidal effects.

The problem of devising a chemical test or group of tests to ac-
count quantitatively for the insecticidal effects of sprays or dusts made
from *Derris,* etc., has presented great difficulty. The total material
extracted by a solvent such as ether, i.e., the so-called total resins, is
not a valid measure, for the ratio of the most toxic constituent,
rotenone, to the other compounds varies greatly among the various
genera and considerably among the species of a single genus and even
among subspecies. Thus the ratio of everything giving a red color
with alkaline sodium nitrite followed by addition of sulfuric acid, i.e.,
rotenone plus rotenoids (45, 46) to rotenone, falls within the range
of 2.0 to 2.4 for *Derris* and *Tephrosia* and 1.2 to 1.6 for *Loncho-
carpus* according to limited tests by Arant (47). Examination of
three subspecies of *Derris elliptica* confirmed this analytical ratio but
showed the ratio of "non-rotenone" toxicity to rotenone toxicity
toward house flies to vary from 0.77 to 1.75 (48). For two species
of *Lonchocarpus* the latter ratio was 0.16 and 0.43. These data illus-
trate the serious error involved in using only rotenone content as the
basis for the evaluation of products from different plant species and
particularly from different genera.

Complete separation of the several constituents is impractical,
but a useful distinction may be made between rotenone (as deter-
mined by crystallization from carbon tetrachloride), toxicarol frac-
tion (the total resins dissolved by 5 per cent alkali solution, in which
the three hydroxy compounds are soluble), and deguelin fraction
(the resins left after removal of the rotenone and toxicarol fraction).
Martin (49, 50) has shown that the "rotenone equivalent," given
numerically by the percentage of rotenone plus one fifth of the per-
centage of deguelin fraction plus one fifteenth of the percentage of
toxicarol fraction, correlated very closely in the case of four different
species of *Derris* with their respective toxicities to the chrysanthemum
aphid and to the sawtoothed grain beetle. Whether this procedure
will be valid for products from *Lonchocarpus* and other genera ap-
parently has not been determined.

For the practical purposes of fixing prices of imported roots and
of standardizing concentrations it is customary in the United States
to take into consideration only the rotenone content. As shown above,
this practice gives a higher rating to *Lonchocarpus* roots since in

general their nonrotenone content is lower than that of *Derris*. In the British Empire rotenone and total ether extract are usually considered. It will be obvious from the foregoing scanty discussion that correlation of biological activity with chemical analysis is not in a satisfactory state, especially when products from different plant genera are concerned. Since uses of these materials are being extended to the control of numerous agricultural pests whose relative susceptibility to the various insecticidal constituents has in many instances not been determined, probably a simple empirical method for standardizing is all that is possible at present. For more detailed consideration of this matter reference may be made to Roark (51) and Jones (52).

The number of plant species known to contain rotenone or rotenoids is quite large, sixty-eight being reported by Jones (53). These included twenty-one species of *Tephrosia*, twelve of *Derris*, twelve of *Lonchocarpus*, and ten of *Millettia*. It is important to note that while the insecticidal constituents occur chiefly in the roots, they have been found in various parts of the plants, frequently in the seeds (54). Before the war interrupted commerce, the chief source of *Derris elliptica* and *D. malaccensis* was Malaya and the Dutch East Indies, though increasing amounts of *Lonchocarpus* spp. were coming from South America and this has greatly expanded of late. Many local names are used for the latter materials, e.g., "timbo" for *L. urucu*, "cube" for *L. utilis*, and "barbasco" for *D. elliptica* from Peru. A comprehensive list for different species of *Derris* was given by Roark (55).

It was found several years ago that *Tephrosia (Cracca) virginiana* or devil's shoestring, which grows wild over a large area of the southern states, contains rotenone in the roots. The content is highest at blooming season (56). The amount is low, however, in comparison with commercial material. Great variations exist between strains growing in different localities or even in the same region and the majority of plants are devoid of toxic components. The insecticidal constituents appear to be controlled primarily by inheritance so that by proper breeding an improved strain may be developed. Thus a certain parent contained 3.63 per cent rotenone and 11.69 per cent chloroform extractable material and the seedling progeny averaged 4.39 per cent rotenone and 13.07 per cent chloroform extractable material (57). However, unknown environmental factors also have an effect. Roots of a parent plant grown in Texas contained an aver-

age of 1.26 per cent rotenone but progeny from crown divisions grown in Georgia and in South Carolina contained 1.92 and 0.52 per cent rotenone, respectively (58). Obviously, there is much to be learned before commercial development can be considered.

The biochemical role of rotenone and the rotenoids in *Derris* and other plant genera is uncertain. The rotenoids present in the seeds first increase in amount and then diminish to a very small residue within a few weeks. Thereafter there is a steady production especially in rapidly growing regions such as the nodes (59). Very fine rootlets contain little rotenone or rotenoids, large roots somewhat less, and roots 2 to 5 mm. in diameter have a maximum amount as calculated on dry weight basis. Starvation, e.g., by severe pruning or exclusion of light, results in almost complete depletion of starch but no rotenone disappears (60), and hence it is not a reserve foodstuff. Of course, on a dry weight basis, rotenone appears to increase during starvation of the plant.

The milky sap obtainable from fresh *Derris* root contains globules of resinous material in which rotenone and rotenoids are dissolved or dispersed. The globules are stabilized by saponins also present in the sap (61). This makes possible the use by primitive peoples of derris "milk" for stunning fish.

The ground seeds of sabadilla, *Schoenocaulon* spp., have been used for centuries for control of parasitic insects such as lice. Recently much interest has been aroused in *S. officinale* which grows in Central and South America. The chief constituents are several alkaloids and an oil. When the seeds are freshly ground and used either in a dust or in a kerosene extract, little toxicity to insects is found. However, activity may be increased by storing the seeds for a considerable period, by extraction at temperatures of about 150°C., or by mixing the powdered seeds with alkaline substances before extraction. It appears that the alkaloids occur in combination, probably with organic acids, and only exert their true toxic properties when liberated from such combination. Treatment of the powdered seeds with alkalis such as sodium carbonate or lime is the preferable method of activation (62, 63). The insecticidal power of a representative sample may be indicated by the fact that a 1 per cent mixture of finely ground sabadilla seed in lime was slightly more toxic to the milkweed bug, *Oncopeltus fasciatus,* than a 0.1 per cent pyrethrin dust.

Trial of the separate constituents, separated chiefly by chromatographic adsorption on alumina, has revealed differences in their rela-

tive toxicities to different insects. Thus, against the milkweed bug or the red-legged grasshopper, decreasing toxicity is shown by cevadine ($C_{32}H_{49}O_9N$), veratrine (the mixed alkaloids), and veratridine ($C_{36}H_{51}O_{11}N$), either as a dust or kerosene spray (64). But in kerosene sprays used against the house fly the order was reversed and differences were large in both instances (65). Since the structures of the toxic compounds are imperfectly known as yet, no explanation of this puzzling behavior is at hand. The toxicity of these materials is high, e.g., kerosene solutions greatly surpass DDT against the milkweed bug and pyrethrins against the house fly. An objection to sabadilla is the sternutative effect of some volatile component.

Another plant which has received attention during the war period as an insecticide is the yam bean, *Pachyrrhizus* spp. Use of the seeds for this purpose has been mentioned at intervals for many years but the first accurate identification of a toxic principle was made by Hwang (66), who found the most toxic portion of the beans to give the color test for rotenone. More detailed analysis (67) showed the ground seeds to be more toxic to silkworms than cube root containing 4 per cent rotenone. Since the seeds contained only a fraction of 1 per cent rotenone their toxicity must be due mainly to another compound. Elaborate anaylsis of fractions of the benzene extract by chromatographic adsorption in an alumina column (68) resulted in seven fractions, three of which showed marked toxicity to silkworms and one to Mexican bean-beetle larvae. The one toxic to both insects was rotenone, another was closely related to elliptone, and the third may be related to tephrosin. Since a crop of yam bean may be harvested each season—and the plant seems to be well adapted to cultivation in this country—it would seem that further chemical, toxicological, and horticultural study is amply justified.

Before leaving the insecticides of plant origin reference should be made to an experimental study of the insecticidal properties of some 150 species of plants (69), and to comprehensive reviews of this subject, one from the United States (70), and two from the British Empire (71, 72).

Synthetic Organic Compounds as Insecticides

Several thousand organic compounds have been tried against a wide variety of insect pests. This testing has been done in part during routine trial of materials synthesized for other purposes but an increasing number of workers are preparing compounds especially for

insecticidal purposes since only in this way can the effect of variation in structure be studied systematically. An indication of the numbers involved is given by a list of 112 organic compounds of iodine, none of which has come into use (73), and by a list of over 1,500 synthetics which have been patented as contact insecticides, usually with the idea of replacing pyrethrum (74). No attempt will be made here even to list the general classes on which work has been done but a very few of the most interesting and important compounds will be considered briefly.

The organic cyanide, trichloroacetonitrile, Cl_3CCN, was introduced in Germany a few years ago as a fumigant for dwelling houses and warehouses (75). It is a liquid, boiling at 85°C. and much less toxic to humans than hydrogen cyanide. In addition, its powerful effect as a lachrymator prevents anyone remaining in a concentration high enough to do harm. In this country it has given excellent results in killing bedbugs, carpet-beetle larvae, and flour beetles at a dosage of 2 lbs. per 1,000 cu. ft. per 6 hr. exposure (76). An added advantage is its rapid dissipation so that fumigated quarters may be reoccupied with very little delay. It is equal to ethylene oxide in effectiveness against grain insects and decreases germination of seeds much less (77). The thiamine content of fumigated grain is not altered. Against bedbugs, the order of efficiency in the presence of blankets and similar material is hydrogen cyanide > trichloroacetonitrile-carbon tetrachloride mixture > trichloroacetonitrile > 1,1-dichloro-1-nitroethene (78) > ethylene oxide (79)

$$[HCN > Cl_3CCN—CCl_4 \text{ (50 per cent of each)} > Cl_3CCN > Cl_2NO_2CCH_3 > \underset{L_O⅃}{CH_2CH_2}].$$
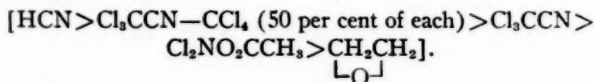
The related compounds monochloroacetonitrile, CH_2ClCN, and acrylonitrile, $CH_2 : CHCN$, are each superior to methyl bromide, ethylene oxide, or carbon bisulfide for control of the grain insect, *Tribolium confusum* (80).

The high toxicity of nicotine and certain related compounds discussed earlier has prompted search for synthetic substances of similar potency. Earlier work had shown the dipyridyls to be highly toxic to several species of insects but no practical application has resulted. Recently, numerous simpler derivatives have been studied. Addition of one or more methyl groups to pyridine has no effect but a L-amino group causes considerable increase in the kill obtained with aqueous

sprays applied to the mite, *Tetranychus telarius*. The increase in effectiveness roughly parallels change in boiling point (81). However, increase in length of straight alkyl chains in either the 2 or 4 position of pyridine increases the effectiveness in aqueous sprays against *Aphis rumicis*. A peak apparently is reached at about eight carbons (82). As a fumigant against the grain beetle the peak is at about four carbons (83).

Another type of organic nitrogen compound, the dinitrophenol derivatives, continue to find wide usefulness. Over fifty years ago a crude dinitro-*o*-cresol was produced in Europe and used against the nun moth which is a serious forest defoliator. It did not become popular largely because of injury to the trees. Lately it has been brought forward as a contact and stomach poison for control of the destructive forest and field may beetles of Europe (84). A very interesting recent development in South Africa is the use of dusts containing dinitro-*o*-cresol against migrating swarms of locusts (85).

Substitution of other groups for the *o*-CH$_3$ of dinitro-*o*-cresol leads to changes in the chemical and physiological properties. Thus the acidic character, water solubility, and toxic effects on plants decrease as the carbon chain lengthens. Toxicity to fifth instar silkworms reaches a maximum in the *n*-hexyl or heptyl derivative and the cyclohexyl compound (frequently called DNOCHP) is nearly as effective against a variety of insects (86). Its toxicity to mammals is fairly low (87). This compound, 2,4-dinitro-6-cyclohexyl phenol, has been very thoroughly investigated for use against mites which infest citrus groves. A 1 per cent dust has given good results against the citrus red mite (88). Various commercial preparations of this material are in use in water and in oil sprays for application during the dormant season against mites and overwintering aphid eggs. Greater safety to plants is secured by forming the dicyclohexylamine salt of DNOCHP which is safe to use as a 1 per cent dust with oil as sticker for control of citrus thrips and the crawler stage of the red scale (89). As a substitute for pyrethrum dust, a mixture of DNOCHP with sulfur has given control of the potato leafhopper (90).

Among organic compounds of sulfur, the thiocyanates, RSCN, have been in use as insecticides for about fifteen years. The best known are the lauryl and the butyl carbitio (β-butoxy-β'-thiocyano-diethyl ether) derivatives which have the formulas $CH_3(CH_2)_{10}$ CH_2SCN and $C_4H_9OCH_2CH_2OCH_2CH_2SCN$, respectively. These

have strong ovicidal action (91) and under closed conditions may also act as fumigants (92). Butyl carbitol thiocyanate, marketed as a 50 per cent solution in kerosene under the name Lethane 384, has been most widely used in sprays for control of flies, mosquitoes, bedbugs, etc. At concentrations of 2 to 3 per cent in kerosene it gives a fairly quick knockdown and compares well with the ordinary pyrethrum sprays in total kill. During the pyrethrum scarcity, Lethane 384 and terpene thiocyanoacetates (93) are the chief toxic ingredients in sprays available to the public. The advent of DDT for household sprays enables a decrease in the use of the thiocyanates but they are still valuable for a rapid knockdown and compete with the pyrethrins for this use (94). An application of special interest is for control of head lice which became a great pest under crowded conditions in war-torn countries. Excellent results have been obtained with a mixture of butyl carbitol thiocyanate and β-thiocyanoethyl laurate (95). In the series of esters formed from a thiocyano substituted acid increase in size of the alcohol radicle results in higher toxicity toward body lice and bedbug eggs (96). The ethers also have desirable properties, e.g., the 6-thiocyanohexylether of 1,3,5-xylanol, $SCN(CH_2)_6OC_6H_2(CH_3)_3$, is highly toxic to flies, freely soluble in kerosene, and devoid of the odor characteristic of many organic thiocyanates (97). A very large number of organic thiocyanates have been tested as insecticides, over three hundred having been patented in the United States up to 1941 (74).

The compound 2,2-bis-(p-chlorophenyl)-1,1,1-trichloroethane, $CH(C_6H_4Cl)_2CCl_3$, often called dichloro-diphenyl-trichloroethane or popularly DDT, has attracted far more attention than ever was given to any other substance used for control of insect pests. Its dramatic appearance during a time of crisis, the screen of military secrecy thrown over every aspect of its nature or uses, and the resultant flood of well-nigh incredible stories combined to raise public interest to a new high for such a subject. Most entomologists were very sceptical regarding the early reports from Europe, such as the claim that a lightly sprayed wall remained lethal to house flies for over three months (98), though subsequent experience has shown that these statements were usually conservative. Entomologists of the J. R. Geigy Company of Basel, Switzerland, in the course of an extensive but rather empirical search for better mothproofing chemicals found toxicity associated with a number of compounds containing two p-chlorophenyl groups. Eventually the 1,1,1-trichloroethane derivative was tried and found

to be superior to all others both for clothes moths and for many other insects such as flies, the Colorado potato beetle, the vine moths, and the cherry fruit fly (99 to 102).

DDT was first prepared over seventy years ago (103) but its chemical inertness offered no hope of finding a use and no mention occurs in the literature until the Swiss workers' reports of 1942. The original preparation was from chloral (or the hydrate) plus chlorobenzene according to the reaction: $CCl_3CHO + 2HC_6H_4Cl \rightarrow CCl_3CH(C_6H_4Cl)_2 + H_2O$. The reactants must be shaken together in the presence of a good dehydrating agent such as strong sulfuric acid. The p,p'-chlorobenzene derivative makes up about 70 per cent of the technical product and is the most effective as an insecticide but about 20 per cent of the o,p'-isomer occurs, as do also smaller amounts of a dozen or so other compounds (104). For routine standardization it is impractical as yet to analyze for any constituent but the setting point is a fairly accurate indication of the composition. This was originally fixed at not less than 88°C. and recently it was raised to 89°C. A so-called "purified" grade must have a setting point not below 103°C. Pure p,p'-DDT melts at 108.5°C.

Technical DDT varies in physical properties from a substance with a gray cheesy consistency to a fine white powder. It is almost insoluble in water (less than 1 p.p.m.) and dissolves in other liquids at ordinary temperature in the following amounts per 100 cc.: ethyl alcohol, 1.5 gm.; dichlorodifluoromethane (Freon 12), 2 gm.; kerosene, 4 to 8 gm.; fuel oil No. 2, 10 gm.; ether, 27 gm.; xylene, 56 gm.; and cyclohexanone, 100 gm. The particles tend to coalesce, so fine grinding is impossible except in the presence of a diluent. Much attention had been paid to the formulation of active preparations of DDT in solutions, emulsions, and dusts for many varied purposes (105).

The first spectacular triumph of DDT on a big scale occurred during the winter of 1943–44 when a growing outbreak of typhus fever in Naples was completely suppressed by the simple procedure of dusting each inhabitant with an ounce or two of 10 per cent powder. For use in the armed forces it was more practical to dip underwear in solutions of DDT in a volatile solvent or in an aqueous emulsion (106) and this was standard practice in certain theaters of action (107).

Since several of mankind's worst diseases are transmitted by mosquitoes, the discovery that DDT is a powerful and persistent poison to both larval and adult stages was an event of well-nigh inestimable

significance. As a larvicide, use has been made of DDT in dusts, oil solutions, aqueous emulsions, and aerosols, and these have been applied by methods varying in simplicity from dropping a little oil solution into a rainbarrel to spraying square miles of jungle from a bomber. Regardless of the method used, an astonishingly small amount is effective. Thus in Alabama an average kill of 96 per cent of *Anopheles quadrimaculatus* larvae resulted from use of 0.1 pound DDT per acre applied from a boat as an emulsified 2.5 per cent solution in kerosene; and 90 per cent control was obtained by applying 0.05 pound DDT per acre from an airplane as a 5 per cent dust (108). When shipping is a serious factor, as it always is in time of war, concentrated solutions have an advantage. These may be made up in xylene, gas liquor, polymethyl naphthalene, etc., usually with some kerosene or light oil. Even in regions of dense vegetation excellent larvicidal action resulted from application of 0.2 pound dissolved DDT per acre (109). *Aedes* and *Culex* larvae are also highly susceptible to DDT, though somewhat higher doses appear to be required (110).

The persistence of toxicity from DDT applied to water surfaces varies greatly. Dusts on undisturbed water remain effective for many weeks but under practical use they tend to be blown aside by winds or carried away by water currents. Oil emulsions and, to a lesser extent, solutions pass into the water and if much colloidal matter is present, the DDT soon becomes ineffective (111). Similar results were obtained in the African Gold Coast in work with *Anopheles funestus* larvae (112). The reason for this behavior is not known as yet.

DDT, either as a solid or in solution, e.g., in kerosene, is considerably less toxic than pyrethrum to adult mosquitoes but the lethal action persists very much longer. Extensive tests in Arkansas proved that rooms treated with 208 mg. DDT per square foot of interior surface applied in kerosene or in emulsion permitted no mosquitoes to remain alive for twenty-four hours over a period of at least five months. The effect was decreased only slightly with 56 mg. per square foot (113). Although the nature of the treated surface is of some importance, especially if it is very porous, one application of 200 mg. per square foot may be depended upon in northern malarial regions of the United States and two are needed in the South (114).

It is impossible even to mention many important contributions on the use of DDT for mosquito control but perhaps the judgment of

most workers is adequately summed up in the words of Simmons (115): "The realization of the efficacy of DDT in malaria control has been a development which is indicated to rank in importance with the discovery of the therapeutic value of quinine and the incrimination of the mosquito in the dissemination of the malady. Exemplification of this hypothesis is manifest in the current conviction of many malariologists that malaria eradication is now in the realm of distinct possibilities." Since other genera are only slightly less susceptible these high hopes may be extended to other diseases, for example, yellow fever.

In the ancient problem of the house fly, DDT offers much help. Toxicity is extremely high—one estimate is that the fatal dose is one millionth of a microgram, i.e., 10^{-12} gm. (116). Symptoms develop much more slowly than with pyrethrum so DDT alone in kerosene is not a good fly spray but when supplemented with pyrethrum or other material of similar insecticidal properties a quick acting, highly toxic, and long lasting product is obtained (117). It may be used as a space spray and also on surfaces around doors, windows, etc., where flies tend to congregate. A particularly interesting development is incorporation of DDT in paints. High and persistent toxicity has been claimed for low concentrations in the so-called water paints which contain but a small amount of oil. In the regular oil paints the hard, smooth surface prevents contact with most of the incorporated poison (118). In the struggle against numerous other insects of medical or veterinary importance DDT is useful in various degrees, e.g., bedbugs are considerably more resistant to it than to pyrethrum (119) but as a practical material for such places as ships, barracks, and jails it gives excellent results. Further discussion is impossible here but for an excellent résumé of DDT in problems of medical entomology, reference may be made to Buxton's article (120).

Investigations on DDT for the control of agricultural insect pests were handicapped during most of the war period by scarcity of material but a flood of reports is now appearing. In brief, results at least as good and usually better than those resulting from use of the traditional materials have been secured with a wide variety of plant-infesting insects. To illustrate the wide variety of insects affected, the following may be mentioned: fruit insects (121), Japanese beetle (122), corn earworm (123), European corn borer (124), forest insects (125), grasshoppers (126), truck crop and garden pests (127), potato insects (128), cotton insects (129), and red scale (130). A

general review of similar work in Great Britain is given by Chambers
et al. (131).

An insecticide toxic to many kinds of insects is certain to reduce
the population of the many beneficial species which are a continuous
natural check to the injurious ones. This disturbance of the natural
balance of nature is especially outstanding in the case of DDT and
has led to hesitation and even opposition to its use on the part of
many careful investigators, especially in cases involving application
to large areas such as forests. The validity of these fears has been
exemplified by outbreaks of mites in apple and pear orchards follow-
ing use of DDT for control of the codling moth. Likewise certain
aphids have given unusual trouble when their predators were killed
by DDT. As in all such cases the solution lies largely in proper tim-
ing of the application of the toxicant so as to do minimum harm to
the beneficial species. In the case of the honeybee little injury results
if use of DDT is avoided during the short time of nectar flow (132).

Before any substance can receive recognition as a practical insecti-
cide it must be examined thoroughly for acute and chronic toxicity to
man and domestic animals. In the case of DDT this matter has been
investigated in this country in the laboratories of the Food and Drug
Administration, in those of the National Institute of Health, in the
Kettering Laboratory of the University of Cincinnati, and to a less
extent in several other institutions. A number of European labora-
tories also have worked on the problem. Fortunately excellent agree-
ment has been reached and the early fear that such a powerful insecti-
cide must be very toxic to mammals has been proved largely false.

When administered in corn or olive oil, which promotes absorp-
tion from the digestive tract, the LD 50 (the dose causing 50 per cent
of fatalities) for rats is about 150 mg. per kg., for rabbits about
300 mg. per kg., and for guinea pigs about 400 mg. per kg. The
values are much higher if the DDT is taken in aqueous suspension
or mixed with ordinary food (133). A female goat given 1500 mg.
per kg. (75 gm. per 110 lb. body weight) in cornstarch developed
typical tremors but recovered, whereas another given 2750 mg. per
kg. (117.5 gm. per 94 lb. body weight) became prostrate in fifty-two
hours (134). These are very large doses compared to the LD 50
figures for many insecticides and they indicate but slight danger of
acute toxic effects from swallowing DDT.

Chronic effects from continued intake are produced in guinea pigs
by DDT at 1000 p.p.m. in the diet, in rats and mice by 500 p.p.m.,

and in chickens by less than 500 p.p.m. (135). Continued daily doses of 25 mg. per kg. body weight given in olive oil to rabbits caused no outward symptoms but within a few weeks led to serious damage of the liver (136). DDT was eliminated in the urine to the extent of from 5 to 8 per cent of the above intake. Cows, horses, and sheep given DDT in their feed at the rate of 100 to 200 mg. per kg. body weight per day suffered loss of appetite within a few days and some of the cows developed tremors of the hind legs. Necrosis of the liver and hemorrhages of the heart and intestines were uniformly found upon autopsy (137). It will be obvious that ingestion of DDT at these relatively high levels causes serious injury but no ill effects appear to have been reported at lower levels such as from 5 to 10 p.p.m. in the diet but the possibility of loss in weight or decrease in normal gain due to decreased appetite is of very great importance.

The presence of DDT in dusts, mists, and aerosols which may be drawn into the lungs during respiration offers another possible hazard. Dogs exposed daily for forty-five minutes over a period of five weeks to an aerosol formed by discharging 33 mg. DDT per l. of chamber volume suffered no outward toxic effects. Three exposures per day for the same period caused no DDT poisoning symptoms but the solvents caused fatal pulmonary injury. Experimenters exposed daily to aerosols of similar concentrations for prolonged periods failed to develop symptoms of injury (138). Dusts are mostly caught in the nose and upper throat and fail to reach the lungs.

The harmlessness of powders containing DDT when applied to the skin is proved by their extensive use in Naples, Egypt, and elsewhere. An exception to the general lack of toxic effects is given by oil solutions, for absorption of DDT through the skin occurs fairly readily from such preparations. Thus toxic symptoms and even death have occurred in several animals following cutaneous application of oil solutions (138, 139) and a case of poisoning with slow recovery of a laboratory worker who dipped his hands in an acetone solution of DDT has been reported (140). In all these instances exposures were excessive but it is doubtless a wise precaution to avoid application of oily solutions of DDT to the skin of either man or animals. Fortunately water suspensions appear to give as good results as oil solutions for control of various biting flies which attack dairy cows and other animals (141).

Because of its solubility in typical lipoid solvents, DDT may be expected to appear in the fatty tissues of animals to which it has been

fed. Thus a dog given 50 mg. per kg. in corn oil for 747 days had 4.94 mg. DDT per gm. of intraperitoneal fat. But administration of dry DDT for 443 days at the rate of 80 mg. per kg. resulted in only 0.39 to 0.67 mg. per gm. of fat (142). Such stored DDT disappears slowly after intake ceases. The substance also appears in the milk of treated animals, especially in the milk fat. Butter from a goat given daily doses of 1 gm. DDT per 8 or 9 lbs. body weight caused tremors in rats to which it was fed. Large single doses have the same effect (143, 144). Since milk and its products are such important foods, especially for young humans and animals, much more work on this very important matter is clearly indicated. A final point of much interest is the observation that bedbugs are rapidly killed by feeding upon rabbits which had received fairly high doses of DDT (145).

Another organic chloride of much promise as an insecticide is hexachlorocyclohexane, $C_6H_6Cl_6$, to which the name "666" has been given (146). This substance has been known as a chemical for a long time and is remarkable for the large number of possible isomers. Of these, four have been isolated and their structures determined with some certainty. It has been found that the so-called gamma isomer accounts for most of the toxicity of the crude product. Hence the term Gammexane has been proposed for this isomer. Its toxicity to house flies is very high—in kerosene sprays nearly ten times that of DDT (147). A special advantage seems to be high killing power for mites which are not affected by DDT (148). Exceptional stability to light and heat indicate that 666 will have a long period of effectiveness but this circumstance may also lead to complications if it is used on edible products.

Soil-inhabiting insects have always been among the most difficult pests to control, chiefly because of the mechanical difficulty encountered in reaching them and because the soil by adsorption or absorption of the toxic material prevents its proper distribution or destroys its toxic properties. Consequently recourse has been had usually to poisoned baits or trap crops which could be destroyed after the pest, e.g., cutworms or wireworms, had come to them. The announcement that a crude mixture of 1,2-dichloropropane and 1,3-dichloropropene reduced the root deformation caused by the rootknot nematode and was an effective fumigant against larvae of the Asiatic beetle (149) stimulated further research on this and similar materials. With pineapple plants the effect of one application at the rate of 150 pounds per acre continued to be noticeable through the third year, doubtless be-

cause of the more vigorous root systems established soon after the plants were put out. In one test plot the yield increased from 3.01 to 18.1 tons per acre (150). This mixture is superior to carbon disulfide for controlling the wireworm, *Limonius californicus,* a severe pest on beans, corn, and tomatoes (151), and it is much pleasanter to use than chloropicrin which is a very volatile tear gas (152). The yields have been increased five to tenfold in regions of heavy wireworm infestation and beneficial results have been obtained even when they were not present (153). This seems to be due to a change in the process of nitrification in the soil (154).

IMPROVEMENTS IN APPLICATION OF INSECTICIDES

Toxic smokes have been used for a very long time as insecticides. This method of application is illustrated by the burning of tobacco leaves or by the use of pyrethrum coils or punks in the Orient. Since actual burning occurs in these cases there is much destruction of the active ingredients. This detracting feature may largely be avoided by spraying a solution such as derris extract in kerosene upon a hot surface from which the solvent instantly evaporates and the toxic material is cast off as a finely divided smoke (155). Pyrethrum, naphthalene, *o*-dichlorobenzene, and many other substances behave similarly. The incorporation of a little oleic or lauric acid or their triethanolamine salts greatly increases the amount of smoke formed and augments the insecticidal ability. Since dispersion is very fine such clouds stay in suspension for a long period and penetrate to every portion of the surrounding space, i.e., they have some of the desirable characteristics of a gaseous insecticide or fumigant and are especially useful against hard-to-reach insects. The term aerosol is an accurate and convenient name for all such fine dispersions.

Use of a heated surface is impractical under many conditions but fine subdivision with or without rapid evaporation of the solvent may be secured by two mechanical means. The first is illustrated by the so-called Phantomyst sprayer which discharges small volumes of spray under considerable pressure through a very small orifice. This machine has been used widely by the British Royal Army Medical Corps (156). The second method is the result of experiments by investigators of the United States Bureau of Entomology culminating in the aerosol bomb, many millions of which have been used by the armed forces.

The principle of the aerosol bomb is that the chosen insecticide is

dissolved in a liquid which at ordinary temperatures has a vapor pressure not less than 50 lbs. per sq. in. and not so high that massive containers are needed. Dichlorodifluoromethane (Freon 12) whose vapor pressure at 80°F. is 86 lbs. per sq. in. is excellent, but several other liquefiable gases are satisfactory (157). A fine needle valve allows escape of the solution which instantly loses the volatile solvent and forms a fine aerosol of the insecticide and any added material such as an oil. The slow rate of settling of aerosols formed in this manner is shown by a comparison of the amounts reaching the bottom of a 29 in. settling chamber. At the end of ten minutes the percentages settled were as follows: mist from an ordinary hand sprayer used at 12.5 lbs. per sq. in., 97 per cent; aerosol formed by spraying a hot plate, 70 per cent; aerosol from a Freon 12 bomb, 34 per cent (158).

Briefly, the desirable properties of Freon 12 are lack of toxicity to man and animals, noninflammability, convenient vapor pressure, low heat of vaporization, and low surface tension (159). It has the disadvantage of low solvent power for most insecticides including DDT. Several auxiliary solvents are suitable for use, e.g., cyclohexanone (160).

The aerosol first developed was based upon a solution of pyrethrin concentrate plus sesame oil in Freon 12. Since part of the pyrethrum concentrate was insoluble in the Freon 12 it was necessary to develop a method for producing a purer concentrate. This problem was solved by use of nitromethane as a solvent (161) and at the same time the product became much less irritating to the nose and throat. This is in line with the conclusion of Martin & Hester (162) that the pyrethrins are not responsible for the dermatitis experienced by some who work with pyrethrum. Two per cent of a 20 per cent concentration was used. Toward the end of the war a mixture of DDT and pyrethrin became available.

The one-pound aerosol bombs issued to the individual fighting men and workers gave excellent results against mosquitoes, gnats, and flies when only a few milligrams of pyrethrins were used per 1,000 cu. ft. While the aerosol bombs were extremely handy under wartime conditions there is by no means universal agreement that they are equally desirable for ordinary use. Comparative tests of DDT-pyrethrin aerosols and concentrated sprays against mosquitoes showed only a slightly higher kill with the former (163) and inferior results against house flies in a room of 2500 cu. ft. volume were obtained when ordinary fly spray was used (164). For agricultural

uses the aerosol bomb is not usually suitable but a number of smoke- or fog-producing machines used for camouflage purposes in the war are being adapted to rapid application of insecticides in very finely divided form.

THE ACTION OF INSECTICIDES

The foregoing account of organic compounds which have been found useful as insecticides has given little, if any, hint of the method by which such substances may be found. It must be admitted that the process has been largely empirical and has consisted of well-nigh endless trying of everything that came to hand. The fruits of this method have by no means all been gathered but there is a widespread feeling that more understanding of what occurs when chemicals adversely affect insects would permit a more discriminating choice of substances for experimentation. This would seem of greater importance even than the search for toxophoric groups for the latter may become strangely inactive when the type of insect or the conditions of use are changed. The mechanism of any toxic action probably lies within one or the other or both of the following conditions: (a) alteration of normal functions or (b) initiation of abnormal functions. Quantitatively such changes must exceed the limits within which a living organism can make accommodation, either with respect to intensity or duration. Since there are numerous methods for observing and measuring either normal or abnormal physiological functions of mammals, the chief difficulties with insects arise from lack of information on normal functions and in overcoming the mechanical problems occasioned by the small size of insects. Visible changes in cells or tissues may provide valuable indications of the site and manner of toxic action, but they may be only incidental to the true lethal process.

Even a casual study of the literature will show that the phrase "mode of action" is poorly defined and ambiguously used and understood. Much of the confusion results from a loose conception of the meaning of toxicity and a continued interpretation of toxicity as only a special case of normal physiological action. There is a great difference in the behavior of a substance to which the organism is adapted and that of a foreign material. Even though the toxic material acts upon a single substance, say an enzyme, to stop its activity effectively, there is no assurance that the distribution of the toxin in the body would parallel that of the enzyme and the over-all result might be hard to interpret in terms of presence or absence of the particular enzyme. If toxicity is measured by alteration of normal

function one must study many functions before one can know the site of action. For example cyanide poisoning causes a multitude of symptoms all of which can apparently be traced to the inhibition of a single enzyme, cytochrome oxidase (165). If toxicity is measured by the dose required to produce death regardless of whether the average individual or any percentage of the number chosen is considered, nothing can be learned of action of a single substance. It is possible, by comparing the relative lethality of several substances, to be able to correlate the total dose with the chemical structures, but whether the changes in lethality are due to changes in toxicity or in effective concentrations at the site of action will be unknown (166). If toxicity is measured by morphological changes as Hartzell (167) has assumed in his studies of neurohistopathology following administration of a number of insecticides, it is uncertain that the tissue in which the change occurs is the one whose malfunction causes death. In any case, many causes which may result in similar pathology and postmortem changes are a constant source of error (168). Even when a single enzyme system is studied lack of specificity may invalidate either positive or negative results; for example, a toxin might inhibit cholinesterase and yet cause death through other reactions. This is exemplified in the findings of Richards & Cutkomp (169) that sodium fluoride inhibits cholinesterase but that several insecticides including nicotine, DDT, and Lethane, which also cause symptoms of nervous derangement, have no effect on this enzyme. When the organ system is simplified results may be different from those obtained in the intact organism; for example Lewis & Richards (170) found that DDT had no toxic action on cells in tissue culture.

It follows that in order to evaluate toxicity as a function of the concentration of the toxic agent one must know the relative importance of the metabolites affected and the actual concentrations of the material at each site of action. In general any departure from this rigorous interpretation of toxicity should place the research in another category, namely, the study of effectiveness. A substance is toxic because it reacts with certain physiological systems; it is effective only when it is toxic and able to reach the site of action in sufficient concentration. A substance may be toxic but not effective under certain conditions but it must be toxic to be effective at all. Many of the divergent results obtained in the study of insecticidal action are due to the measurements being made in such a manner that they are not comparable. Two substances of the same toxicity to the same entity in the

organism may be compared with respect to effectiveness in the same organism. However, a change in organism may change the effectiveness, and hence, they are compared with difficulty in different organisms. It is obvious then, how difficult it is to compare the effectiveness of substances of different toxicity when different organisms are used.

The mode of application of an insecticide is of great importance (171). Nearly all insecticides are combined with a more or less inert carrier which may greatly modify the ease of entry into the insect. The several portals of entry, the integument, the intestinal wall, and the respiratory system differ greatly in their permeability to both insecticide and carrier. It is possible to use the mortality rate of the test insect as a measure of the ease of penetration by the several routes and of the effect of the carrier. Any impairment of the surface lipoid layer of the insect cuticle affects its permeability (172). The mechanical scratching of the insect cuticle by such a material as silica dust may cause death by increasing the rate of loss of water from the body (173). This is of importance when comparing insecticidal dusts containing different carriers.

It is in the study of chemical constitution of a toxin and its effectiveness that the great difficulties of interpretation become most obvious. Most toxicologists are agreed that lipoid solubility increases effectiveness either by increasing ease of penetration or by concentrating the material in the site of reaction. In many complex organic molecules a portion of the molecule may contribute to its lipoid solubility and another portion to its toxic action. It may in some cases be difficult to decide which portion performs which function. In DDT both the chlorophenyl and the trichloroethane group are lipoid-soluble to some extent and Läuger et al. (174) considered that the trichloroethane group confers the lipoid solubility whereas Martin & Wain (175) considered that the chlorophenyl group is the lipoid-soluble portion. If the trichloroethane group confers solubility, then the chlorobenzene group is the toxic portion, whereas if the chlorobenzene group is the lipoid soluble portion, then either the chloroethane group is toxic or, as suggested by Martin & Wain, the toxicity is due to hydrolysis liberating hydrochloric acid. There is the other possibility that the action of DDT is permitted by its molecular configuration (176). Busvine (177) in attempting to reconcile the divergent viewpoints of Läuger and Martin studied a series of analogues of DDT. No correlation of lethality and lipoid solubility was found. Busvine

concluded that the stearic configuration of the molecule was probably
of importance. The remarkable importance of molecular configuration
so well known in physiology is again exemplified by the isomers of
hexachlorocyclohexane (178). The gamma isomer, called Gam-
mexane, is nearly ten times as toxic as any of the others. This fact
has led Slade to suggest that Gammexane acts by substituting for the
configurationally similar compound inositol which is a member of the
vitamin B complex. This theory is greatly strengthened by the fact
that the physiologically active form of inositol, *meso*inositol, has the
same configuration as the gamma isomer (179).

One of the useful methods of eliciting the physiological action of a
substance is by studying its antagonists and synergists. Yeager &
Munson (180) showed that nicotine and DDT are not antagonistic
and do not act at the same site, DDT apparently acting on the motor
neurone. Metcalf & Kearns (181) showed that barbiturates protect
the cockroach from DDT but that curare, chloral hydrate, and
urethane are inactive. They interpret this to mean that no irrevers-
ible damage occurs to the nervous system. Physostigmine increased
the action of DDT but acetylcholine, epinephrine, cocaine, and strych-
nine were ineffective as stimulants. Metcalf & Kearns concluded that
DDT acts by lowering the threshold of synaptic resistance. By study-
ing similar compounds they concluded that neither the trichloroethane
nor the chlorobenzene group is essential for toxic action. Vaz *et al.*
(182) have shown that calcium gluconate protects dogs from the
action of DDT and also relieves the symptoms of DDT poisoning, but
there is no evidence that calcium has the same action in insects.

A mixture of substances may act in a number of different ways as
compared to the action of any of the constituents considered alone.
Bliss (183) defined three categories, independent joint action, similar
joint action, and synergistic action. In independent joint action each
component acts upon a different physiological system whereas in
similar joint action each substance acts upon the same physiological
system in a similar manner so that one can be substituted for the
other at a constant ratio. If the physiological effect of the substances
exhibiting a similar joint action is significantly greater when used
together than when the independent effects are summed, the joint
action is termed synergism. Cox (184) restricted the term synergism
to the cases in which each component is toxic; if a nontoxic compo-
nent increases the toxicity it is an activator. Negative synergism is
antagonism. Finney (185) and Wadley (186) have given statistical
methods for testing for synergistic action. It is difficult to understand

how two toxins exhibiting strictly similar joint action, that is, acting upon the same physiological entity could possibly exhibit synergism unless one component increases the concentration of the other at the site of action.

Synergism as usually defined means that the two substances used together produce a greater effect than the sum of either taken alone. In general the effect produced is the sum of several physiological processes. In the case of insecticides the effect is death which may be occasioned in a great many ways. One would expect that synergistic action (broadly defined) of insecticides would more often result when the substances are not similar and act on different parts of the organism to produce death. Certainly the mode of action of insecticides is too little understood at present to be sure that a similar slope in the dosage-mortality curves indicates similarity of action. It would seem much better, for the present, to speak of toxic and nontoxic activators (187) or to use the more general definition of synergism. In either case synergistic action can be confirmed only if the conditions are kept exactly constant. For example, in the Pect-Grady method of testing insecticides, many factors are important. Among these are the droplet size (188) which is determined by the kind of sprayer; pressure and physical properties of the carrier; vapor pressure of the toxicant which controls the relative contact and fumigant action; carrier activity which determines the rate at which the toxicant reaches the site of action. On addition of an activator the system becomes more complex since the activator is also affected by the carrier and may itself act either at the surface of the insect or at the site of action of the primary toxicant. The foregoing principles must be kept in mind in evaluating the published work on synergism. Space does not permit an analysis of the papers, which must be consulted for the details. An example of the difficulty of proper evaluation of experimental results is shown by two studies of pyrethrin sprays activated by several substances. David & Bracey (189) using *Aedes aegypti* as the test insect showed that sesame oil, isobutylundecylenamide (IN 930), desesaminized sesame oil, and lubricating oil all act as activators of pyrethrins. These substances delayed knockdown and increased kill. Their explanation was that the carriers of low volatility maintained a large drop size resulting in a larger deposit upon the insect. The unexplained delayed occurrence of knockdown in the presence of these activators also increases the deposit. Parkin & Green (190) using *Musca domestica* as the test insect and sesame oil, oleic acid, medicinal paraffin (heavy white oil), and lubricating oil as

activators concluded that the nonvolatile adjuvants had no activating effect and that kill was not dependent upon knockdown.

Following the early work with sesame oil, investigators reported the activating effects of a number of compounds. Thus Pierpont (191) showed that the ethylene glycol ether of pinene (D.H.S. activator) was effective in activating pyrethrum and rotenone and later (192) extended his studies to its activation of an aliphatic thiocyanate, Lethane 384. McGovran & Sullivan (193) showed that methylphenyl-nitrosoamine and 2,4-diamylcyclohexanol activated pyrethrins against the house fly. Haller and co-workers (194) have studied the activating effect of compounds related to sesamin such as pinoresinol, isosesamin, asarinin, and a series of N-substituted piperonylamides. Harvill et al. (195) have shown that piperine is more toxic to house flies than pyrethrum and that a mixture of 0.05 per cent piperine and 0.01 per cent pyrethrins is more toxic than 0.10 per cent pyrethrins. Increasing the side chain attached to the methylenedioxyphenyl group increased the effectiveness of the amide. Synerholm et al. (196) have tested a number of derivatives (esters and amides) of piperic acid and showed that amides derived from primary and secondary alkylamines of three to seven carbon atoms and esters of alcohols of four to six carbon atoms were most toxic. Both amides and esters show an activating effect with pyrethrins. Rice et al. (197) have found that the addition of Thanite (a terpene thiocyano ester) to DDT gives a spray of more immediate effect, that is, increased knockdown and gave as good kill as a comparable pyrethrin spray. Marcovitch (198) found that a mixture of sodium fluoride and pyrethrum was more toxic to roaches than either compound alone. In this case the paralyzing action of the pyrethrum may prevent the roach from cleaning itself of the fluoride. This would not seem to be a case of activation.

The numerous cases just cited give illustrations of so-called synergism though they have been examined mathematically in only a few instances. Whether the components act upon the same physiological system and to what extent the effects can be correlated with changes in concentration of the toxic compounds at the site of action is yet to be learned.

The search for better insecticides must continue because there is increasing demand for higher quality agricultural products and more rigid standards of public health. In this work chemists and entomologists are co-operating to the end that their products shall have maximum toxicity toward insects and minimum harmful effects upon man and animals.

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THE VIRUSES

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During the last ten years the character of virus research has undergone a change. At the beginning of the period the important thing was to get viruses accepted as entities with properties that could be studied by the ordinary methods of physics and chemistry. It was essential to remove them from the sphere of pure pathology where they were in danger of being considered unknowable because they were invisible by the methods then in use. They had to be related, to the greatest extent possible, with the other materials adapted to biochemical study. This phase has passed and the rather hectic and uncritical aspects of the early work can be seen in retrospect to have been useful in directing attention to the main proposition. The claim to have isolated another virus in a pure state no longer excites strong comment, either of incredulity or praise, and no one now doubts that viruses have properties that can be described in biochemical terms. It is sometimes even legitimate to accept the published properties as being those of the virus. The elimination of the old "defeatist" attitude has been accompanied by a tendency towards too ready belief; it is necessary therefore to comment on some misconceptions that have arisen as a result of inaccurate and oversimplified presentations of the subject. It will perhaps be profitable to see whether any principles can yet be clearly identified, whether any generalisations are yet justifiable, or whether any of those that are commonly made are unwarrantable.

The field can be considered in terms of three questions. Can viruses be considered a group that is chemically and physically distinguishable from all other biological material? Are there general chemical differences between plant, bacterial, and animal viruses? Can any classification of viruses be attempted?

If we confine our attention to statements that have been confirmed by independent workers it is clear that viruses form a group covering a very wide range of particle weights. The virus which causes silkworm jaundice (1, 2) seems to be the smallest for it has a sedimentation constant of 10 to 17 S while the larger viruses sediment a hundred times as rapidly and are no longer sharply differentiated

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from the rickettsias or bacteria. It is clear that particle size alone is not sufficient to differentiate viruses from other tissue components, for macromolecules or particles with sedimentation constants in this range are now recognised as components of normal fluids such as milk (3) or the allantoic fluid from hen eggs (4). They are also found in tissue extracts made by methods comparable to those used in virus extraction; Claude (5, 5a) suggests that the particles made by the differential centrifugation of extracts from lymphosarcoma tissue may be analogous to the mitochondria or microsomes of normal tissue. From nuclear extracts he has made preparations of chromosome fragments (6). The cell wall may also contribute particles in the size range associated with viruses for a sedimentable lipoprotein has been made from mammalian red cells (7), and there is reason to think that it is derived from the stroma. Other workers (cf. 8) have described the association of several enzymes with the readily sedimentable components from many tissues, and it is probable that some of the enzyme activities that have been attributed to viruses are due to incomplete separation from these normal components. Clearly particle size alone will not suffice for the recognition of a virus and clearly also it is wise to choose as a starting material in a virus preparation a fluid known to be free from other components of similar sedimentation constant.

No chemical differentiation of viruses from normal tissue components is yet possible for, although several viruses appear to be nucleoproteins, nucleoprotein is a common constituent of the normal macromolecules and these are often antigenically active. Viruses can therefore only be recognised by their ability to bring about defined physiological changes in the host and not by their own intrinsic properties. Their host range is wide, covering animals, bacteria, and angiosperms but it is interesting to note that viruses are not known to infect gymnosperms, pteridophytes, or bryophytes.

A simple tabulation of the properties of plant and animal viruses shows clear differences between them but before any significance is attached to these differences it is necessary to consider whether the viruses that have been studied are a properly representative sample from the whole field. With animal viruses the choice has been controlled by pathological considerations for vaccine therapy is so well established that it is obvious that a knowledge of the properties of a virus will increase the probability of cure. Choice is not, therefore, primarily influenced by physical or chemical considerations but in-

tensive study is given to those of serious medical importance, such as influenza or poliomyelitis, or to those in which the first stage of the fractionation is a normal commercial process, as in vaccinia. Social and economic factors, therefore, have controlled the choice of experimental material, and there is no reason to think that these factors are in any way correlated with the intrinsic properties of the viruses. With plant viruses, on the other hand, the choice has been free; this means that a rough chemical classification has in fact been introduced, for the more stable and abundant ones have been selected for study. Fairly definite statements have been made on the properties of about twenty plant viruses or virus strains. Both the original choice of virus and the type of phenomenon sought have been limited by the interests of the workers without regard to economic importance. Any uniformity which appears to run through the group of plant viruses is at least as likely to be due to this initial selection as it is to be a genuine property of the viruses. The generalisation that "plant viruses are simple nucleoproteins" appears to lack foundation and the more speculative deductions from it (9) should be accepted with caution. More workers are engaged in the biochemical study of animal than plant viruses, and they have undertaken careful and elaborate pathological and serological investigations that are without parallel in plant virus research. It is this concentration that has made progress possible in these complex but practically useful fields. The dissimilarity between the properties of extracts of animal and plant tissues acts as a further factor controlling the types of virus that can be studied. Extracts from many normal or neoplastic animal tissues are well adapted to the direct performance of serological tests. Leaf extracts, on the other hand, undergo a variety of changes in their colloidal state, generally involving precipitation, and these may render direct tests equivocal. This is a factor which limits, or has limited, plant virus study to those viruses that withstand the treatments necessary to get stable extracts. An extreme case of this phenomenon is seen in the strawberry (10) where extracts from infected leaves not only appeared to be free from virus but contained sufficient tannin to precipitate other proteins added to them. If a plant virus exists with properties similar to those of the influenza viruses, it is very unlikely that it would have appeared in any of the leaf extracts made by methods used so far.

From the preceding paragraph it is clear that so many factors influence the direction of plant and animal virus research that differ-

ences in the conclusions may not reflect genuine differences between the two groups of viruses. It is highly desirable that, in so far as the need for somewhat different facilities for their study can be met, the study of viruses attacking plants, bacteria, and animals should be carried on in parallel.

No satisfactory system of virus classification has so far been proposed. This is hardly surprising when one remembers that more than two hundred plant viruses have been described but only a tenth of them have been subjected to chemical or physical examination. Biological classification depends largely on morphology and, with the smaller viruses, morphology becomes simply a study of chemical and physical properties. The classification of these viruses becomes, therefore, one branch of the more general theme of protein classification and only those that have been studied in some detail can, in a strict sense, be classified. Several attempts at classification have, however, been made; these have been criticised cogently by Bawden (11) and McKinney (12). The latter at the same time proposes another system which, while more closely in accord with accepted botanical conventions, pays as little attention as the earlier systems to the intrinsic properties of the viruses. Bawden (13) points out that the earlier schemes are systems of labelling only, and he proposes the beginnings of a true classification which is not based only on symptomatology but on serological relationships, the properties of purified preparations, and the ability of one virus culture to protect the host plant against subsequent infection with another. He wisely refrains from a wholesale renaming of all the known viruses and limits the arrangement to thirty viruses divided into twelve genera so that there is an obviously closer relationship between one virus and the other members of its genus than there is between that virus and any other genus. Intergeneric differences are not all of the same order and some suggestions are made for the grouping of the genera into families. On this system most viruses remain in an unclassifiable limbo; this is not a defect of the system but an accurate statement of the limitations of our knowledge. Such a system can be extended to the animal viruses but poliomyelitis virus is the only one with properties at all similar to those of any of the plant viruses. It resembles those in the tobacco mosaic, potato X and potato Y groups, for purified preparations show a tendency towards linear aggregation and contain about 0.6 per cent phosphorus (14). Detailed classification will only be possible when we have information about many more viruses.

Influenza Viruses

In the period under review notable progress has been made in the study of the viruses causing influenza; several strains of both virus A and B have been used and a large measure of agreement has been reached by different groups of workers. Most preparations have been made from the allantoic or amniotic fluids of hen eggs in which the virus has been allowed to develop for one to three days but concentrates (15) have also been made from infected lungs and, when purified, they resemble the chick embryo preparations closely (16). Differential centrifugation is, with this as with so many other viruses, a convenient method of separation from most of the inert matter in the original virus-containing fluid; it has been supplemented by adsorbtion on calcium sulfate, on calcium phosphate, on the precipitate that separates when allantoic fluid is frozen and, more specifically, on chicken red blood corpuscles (17). The yield from allantoic fluid is about 20 mg. per l. Preparations made by adsorbtion on chicken cells followed by release and ultracentrifugation are relatively homogeneous but a critical study of the spread of the boundary during ultracentrifugation and of the variations in image size in the electron micrograph (18) shows that the preparations are polydisperse with a symmetrical distribution of sizes about the mode. This has been interpreted (18) as evidence that the full properties of the virus are associated with particles having a range of sizes but this does not seem to be a necessary conclusion. The three properties most characteristic of influenza virus are: infectivity, ability to agglutinate red cells, and complement fixation. Friedewald & Pickles (19) find that the apparent sedimentation constant of the infective principle is greater than that of the haemagglutinin and that material with a still smaller sedimentation constant can still fix complement. In these experiments the virus was ultracentrifuged in a sucrose gradient made by adding successively more dilute sucrose solutions, all containing the same concentration of virus, to the centrifuge tube. In comparable experiments in a centrifuge cell fitted with a moveable partition Lauffer & Miller (20) found no difference between the sedimentation constant of the infective and agglutinating principles. The discrepancy may be due to the different techniques or to differences in the initial degree of polydispersity in the virus preparations used by the three groups of workers. There is already evidence that some influenza viruses disintegrate *in vivo* (21) and that a slight increase

in the temperature at which the infected egg is incubated (22) causes
a breakdown of virus B to products which may contaminate the final
preparation. Polydisperse preparations may therefore arise from
material that would have been uniform when in the fully infective
state.

In recent work there has been no confirmation of the early (23)
suggestion that infectivity could be associated with particles only 10
mμ in diameter. Values for the sedimentation constants of three
viruses are set out in Table I. As with other proteins the value de-
pends to some extent on the concentration of the suspension on which
the measurement is made; the values given are corrected by extrapola-
tion to zero concentration of virus and also for the change in the
viscosity of the solvent when measurements were made at tempera-
tures other than 20°C. Table I also gives the mean diameter of the
images on electron micrographs of the viruses. The authors quoted
point out that these values for the diameter of the dry particles agree
well with values that can be deduced from the sedimentation con-
stants of the hydrated particles and the densities derived from meas-
urements of the sedimentation constant made in serum albumin
solutions of known density (24). Similar measurements in sucrose
solutions give a higher value for the density but evidence is presented
that strong solutions of sucrose affect the density of the particle, for
the relationship between density of solvent and apparent sedimenta-
tion constant is non-linear (25). The value given by Lauffer & Stan-
ley (26) has been included in the table for it tends to confirm the
others; however, it was found by centrifugation in sucrose solutions,
and it is by no means clear how the authors have derived it from
their published results. These values, taken in conjunction with the
partial specific volume, give a measure of the water content of
the hydrated particle; as Table I shows, between a third and a half
of the volume of the particles in the suspensions on which sedimenta-
tion constants are determined is water. These values are somewhat
higher than those usually given for the hydration of proteins (cf. 27)
and, although the method by which they have been derived appears
to be valid, it should be remembered that unequivocal values for the
hydration of other proteins in solution have not yet been given. The
fact that all egg preparations are probably slightly contaminated (26)
with a normal embryo component (4) of high viscosity makes vis-
cosity measurements inapplicable for this purpose. Without more
knowledge of the hydration of particles in solution and of the changes

brought about by drying, the agreement between electron micrographs and sedimentation constants must remain interesting rather than conclusive. Influenza virus particles are of a suitable size to form excellent objects for electron microscopy and Müller's technique (28) of casting a metal "shadow" of the dried preparation by evaporating a film of metal on to it from one side has given extremely clear and beautiful results (29).

TABLE I

SOME PROPERTIES OF THE INFLUENZA VIRUSES

	Virus A (PR8)	Swine Virus	Virus B (Lee)
Sedimentation constant (S = cm. sec⁻¹. dyne⁻¹x 10¹³)	742 (18)* 700 (19) 722,658 (26)	727 (18)	840 (18) 800 (19) 832 (25)
Mean diameter of electron microscope image in mμ......................	101 (18) 115 (26)	96 (16)	123 (16)
Density in aqueous suspension........	1.104 (24) 1.1 (26)	1.100 (24)	1.104 (24)
Partial specific volume..............	0.822 (24) 0.78–0.85(26)	0.850 (24)	0.863 (24)
Percentage, by volume, of water in the hydrated particles (24)	52	43.3	34.5
Percentage, by weight, of lipid in the dry particle (30).................	23	24	22
Percentage of nitrogen in the dry particle	10.0 (30) 10.35 (26)	9.0 (30)	9.7 (30)
Percentage of phosphorus in the dry particle	0.97 (30) 1.13 (26)	0.87 (30)	0.94 (30)

* Numbers in parentheses refer to Literature Cited.

Chemical composition.—The influenza viruses contain about 1 per cent phosphorus; half of this is in the form of phospholipid (30) and the remainder is associated with the protein. Colour reactions for a desoxypentose are given, and it is generally assumed that the intact particle contains nucleoprotein of the desoxypentose type; this does not seem to have been demonstrated conclusively and Cohen (31) finds that the sedimentable component of normal allantoic fluid, which probably contaminates most preparations, also gives the re-

action. A suggestion that purines or pyrimidines are present comes from the demonstration (32) that light of wave length 265 mμ is a more potent inactivator than light of other wave lengths between 200 and 295 mμ. This cannot be simply interpreted, for other viruses which contain nucleic acid and which show a local maximum in their absorbtion spectra are not preferentially inactivated by light of that wave length (32).

More carbohydrate is present in the non-lipid fraction than would correspond to the phosphorus if this were present as nucleic acid. The nature of this material has not been closely investigated but pentose seems to be absent. In Table I the figures that Taylor (30) has published for the lipid content of three influenza viruses are set out; all aspects of the chemistry of the lipoproteins, however, are so uncertain (cf. 33) that further evidence is needed before the whole of this lipid can be accepted as necessary for virus activity. It is clear, however, from these analyses that influenza virus preparations have a more complex composition than the other virus preparations that have been examined. Friedewald (34) has shown clearly that the influenza viruses are antigenically complex and that the different strains of virus A have common antigenic components but nevertheless differ from one another both quantitatively and qualitatively. Although some of the products of virus disintegration are antigenically active (19) no attempt has yet been made to relate these various antigens with the chemically distinguishable components of the intact particle.

Stability.—Measurements of the stability of the influenza viruses show that they, like other viruses, lose their various capacities at different rates and under different conditions. Infectivity is, as usual, the most readily destroyed but clear interpretation is complicated by Knight's observation (35) that virus, no longer able to infect chick embryos or mice after twenty-seven days' exposure in water, was reactivated by the addition of phosphate or borate buffer. This effect is apparently not due to the aggregation or dispersion of the particles. The various factors affecting stability are interrelated; thus the pH stability depends on the medium in which it is measured. Proper control of the medium can only be attained with purified preparations and these are generally made by ultracentrifugation, but this is known to cause partial, irreversible inactivation (30). Furthermore the viruses are more stable when kept at 2 gm. per l. than when kept at 0.1 gm. per l. (36); this makes stability depend on a pre-

liminary concentration. All the viruses are stable at pH 7 but they differ in their stability range; thus the swine virus and the Lee strain of virus B are stable at higher pH values than the PR8 strain of virus A (36), although even this virus is not noticeably inactivated in an hour at 23°C. and pH 11. There is much greater instability on the acid side of the pH of greatest stability and inactivation is rapid at pH 5.5 (36). The isoelectric point is 5.3 (37). Even under optimal conditions in phosphate buffer the stability can be enhanced by the addition of sodium chloride (36) or arginine (38).

The infectivity of virus suspensions is not greatly reduced by phenol (25, 38), chloroform (25), penicillin (39), sulfadiazine (40), or sulfathiazole (25) in concentrations sufficiently high to prevent the multiplication of most bacteria. Complete loss of infectivity is readily achieved with oxidising agents or the salts of copper and silver (25) but this is associated with the loss of the ability to agglutinate red cells and to confer immunity. Many detergents inactivate the viruses (41) and under suitable conditions such products can be used to immunise mice (42). Vaccines have also been made by Stanley (25) and others by treating the virus with formaldehyde or exposing it to ultraviolet light, the conditions of exposure have to be precisely defined, for many workers have found that the viruses readily undergo total inactivation.

Changes brought about by the influenza viruses.—The processes of attachment and detachment that the virus undergoes in the presence of chicken, ferret, human, or guinea-pig red cells are generally referred to as adsorbtion and elution but Hirst (17) has stressed the resemblance between these processes, in which about equal numbers of virus particles and red cells are involved (19), and an enzyme action. Red cells with virus attached tend to cohere and, if the clumps are dispersed mechanically, they quickly reform. After a time however an irreversible separation takes place which leaves the corpuscles no longer able to combine with the virus but the latter can combine with fresh corpuscles. There is no evidence on the nature of the change that is taking place or of the substance that is being destroyed but Hirst (43) suggests that lung epithelial cells are similarly affected and that the exhaustion of a receptor substance is a necessary preliminary to the invasion of susceptible cells. The change may legitimately be called enzymic and it is the only enzyme activity that has been attributed to the virus.

Some information is now accumulating on the nature of the

changes that accompany the infection of the chick embryo. In normal
eggs there is a fall in pH, from 7.9 to 5.6, during the second half
of development but in infected eggs there is either no fall or a very
much smaller one (44, 45). After a few hours' development one of
the influenza viruses can render an embryo insusceptible to infection
by a dose of serologically unrelated virus that would otherwise have
caused infection (46). For this effect it is not necessary that the
virus used in the first inoculation should be capable of causing in-
fection, for protection is achieved with virus inactivated by ultra-
violet light (47), by ageing *in vitro,* or by heating (48, 48a). Non-
infective virus will protect the embryo from subsequent infection by
virus of the same strain. Careful study (47) has brought forward
strong evidence that the protection is due to the inactivated virus
rather than to any other component of the preparation.

These phenomena agree well with the suggestion that there is
a structure in the susceptible cell to which the virus must anchor
itself before infection can proceed. There are similar phenomena in
other fields of virus research; thus one strain of a bacterial virus will,
after inactivation, impede the multiplication of either the same strain
or another in the host bacterium (49). One inactivated virus par-
ticle appears to be sufficient for each bacterium. Non-infective prep-
arations of plant viruses have not been shown to behave in this way
but the mutual antagonism of different virus strains is well known
and the suppression of one virus by another has been described (50).

POLIOMYELITIS VIRUS

Some of the earlier attempts to purify the viruses causing polio-
myelitis have been surveyed by Gard (14). One of these (51), in-
volving extraction of the ground infected brain, partial removal of
the lipids with ether, precipitation with ammonium sulfate, and ultra-
centrifugation, has been modified and improved by him and used also
on faeces from human and animal cases of the disease. In the main
his conclusions have been confirmed (52). In this work human
strains, mouse adapted human strains, and the virus of Theiler's dis-
ease have been used. The yield of final product is about 1 mg. from
200 mouse brains or 200 gm. of faeces.

From all sources the preparations are slightly opalescent and
contain a component with a sedimentation constant of about 150 S.
Electron micrographs show filaments 15 mμ wide. Purified prep-

arations readily aggregate linearly and show anisotropy of flow. In these and other respects they resemble some plant virus preparations closely. Only one preparation, from human faeces, has been analysed but it contained 0.6 per cent of phosphorus as in those plant viruses that can occur in a rod-shaped form. The ultraviolet absorption spectrum of this preparation is compatible with the presence of nucleic acid but there is no other evidence that the preparations contain nucleoprotein (14).

Active virus is excreted by apparently normal people and it is often present in urban sewage although it is not as stable in this medium as in brain extracts or faeces (53). The faeces (54) or intestinal contents (55) of apparently normal animals contain a similar non-infective protein. Gard discusses carefully the relationship between this material and the virus; he suggests tentatively that it is an a-virulent or at least non-neurotropic form and also that it may multiply in the intestinal flora or fauna rather than in the gut wall. The possibility that it may be derived from bacterial flagella is also realised (56).

TOBACCO MOSAIC VIRUS

The properties of tobacco mosaic virus have been reviewed so recently (57), in a manner which seems adequate to the present reviewer, that only papers that had not been seen when that review was written will be considered here.

Lauffer (58) has measured the viscosity, diffusion constant, sedimentation constant, and distribution of particle lengths on several virus preparations made by various methods. The addition of phosphate during the preparation or ageing of the purified product causes aggregation, but even the least aggregated product is inhomogeneous. Thus half of the rods measured on the electron micrographs had lengths above or below the mode of 270 mμ, in spite of the centrifugal fractionation to which the virus had been subjected, and the "spread" of the sedimentation boundary corresponded to a standard deviation of 14 per cent in the particle length. These results do much to promote unanimity over the properties of this virus, for Bawden & Pirie have long maintained that the processes of "purification" cause aggregation of the particles initially present in the fresh infective sap. They have now published more detailed evidence for this point of view (59) and have shown that the normal components of tobacco

sap are themselves potent aggregating agents. By the differential centrifugation of extracts in which this aggregation had been minimised, fractions were made, each of which could be partly or wholly converted into the conventional aggregated material but many of which were not obviously anisometric when fresh. The most slowly sedimenting fractions contain up to 40 per cent of material inconvertible into aggregated virus and these fractions are much less infective than those which are more completely convertible. The complex set of relations in the aggregation and disaggregation of the virus is most simply interpreted by postulating primary virus particles which are unable to exist free in solution but combine quickly either with each other or with other materials in the solution. The average length of the particles in a virus preparation is controlled by the past history and present environment of the preparation; it is a compromise between the forces leading to end-to-end adhesion and those, such as thermal agitation, that tend to break this adhesion. Nothing is known about the forces holding the aggregated rods together nor about the magnitude of those tending to break the rods. A beginning has been made, however, for Frenkel (60) has shown theoretically that above a critical velocity gradient molecules will tend to be disrupted if their extension is proportional to the extending force and ultrasonic disruption of the virus rod has been demonstrated (61). A series of striations crossing the rods can be seen in electron micrographs of some tobacco mosaic rods made by the metal shadowing technique (62), the interval between the striations is not uniform and no suggestion about their nature has been given.

The picture that is presented of a breakable rod which, when broken, leaves polar ends that either join together again or accumulate other material from the environment has some obvious similarities with the phenomena described at the "sticky" end of a broken chromosome (cf. 63). It is perhaps significant that aggregation of this type is confined to viruses with a low nucleic acid content and that there is evidence (64), from a study of the absorbtion of polarised ultraviolet light in a solution orientated by flowing in a quartz capillary, that in tobacco mosaic the nucleic acid lies on the outside of the rod. A break therefore, as is suggested with chromosomes, would leave free protein at the ends; there is evidence that the protein moiety of the virus can aggregate, for Schramm (65) has regenerated rod-shaped particles from it. This analogy may be worth bearing in mind but it would be premature to develop it further.

Other Plant Viruses

Tobacco necrosis viruses.—The symptoms of tobacco necrosis, like those of influenza, can be caused by several different viruses; five of these have been isolated (66) and differences in their physical properties, serological behaviour, and crystal form recognised. One virus, which was sharply differentiated from the others by its greater instability and smaller sedimentation constant (S = 50) has now been studied in more detail (67). No preparation has crystallised when precipitated by ammonium sulfate but purified preparations crystallise readily from water; the pellet that separates on ultracentrifugation appears to be crystalline throughout. With all viruses there is still some legitimate uncertainty over the identification of the infective material with the major component of the preparation; with this virus the uncertainty is accentuated because fractions differing in their rate of solution and bearing different infectivities can be separated from these pellets. X-ray measurements (68) on the crystals of a non-infective derivative of the virus suggest that the particles are spherical and a "molecular weight" of one and a half to two millions can be deduced from them. This agrees with the value derived from the sedimentation constant (69) by making the usual assumptions.

Price (70) has isolated from beans infected with southern bean mosaic a protein with a crystal form and other physical properties similar to those of some of the tobacco necrosis viruses.

Insect-transmitted viruses.—From mosaic infected wheat (71) and oat (72) plants, extracts have been made which give a shimmering precipitate of needle-shaped crystals or fibers on the addition of acid. These preliminary observations are of interest for these viruses are not sap transmissible and they are only transmitted by their vectors and then only after an incubation period. The vector for winter wheat mosaic is *Deltocephalus striatus* and that for oat mosaic *Delphax striatella;* from each leaf hopper, after feeding on infected plants, similar needles could be prepared.

There is now confirmation (73, 74) of the old observation (75) that infective nucleoproteins could be prepared by ultracentrifugation from plants infected with potato virus *Y*. Preparations of potato virus *A* have also been made (73) and these, like the preparations of *Y*, contain about 0.5 per cent phosphorus and the particles appear to be elongated.

Associations between a Virus and its Host

The successful study of the properties of isolated viruses has tended to direct attention away from the older problem of their state and method of multiplication in the host and of the mechanism by which they cause the observed symptoms in it. Wynd (76) has reviewed comprehensively the metabolic phenomena that accompany virus infection in plants but the review shows that in spite of the vast amount of work that has been done in this field few clear principles can yet be distinguished. There have recently been great developments in biochemical ideas and technique and a return to the problem of what a virus does as opposed to what it is seems to be overdue.

A virus is only likely to manifest its presence in a susceptible host if at least part of it is soluble, for otherwise there would be little chance of the damage spreading so far as to become noticeable. This is in fact one important distinction between a virus and a gene for the latter is, by definition, anchored. It is well known that part of the virus exists in insoluble states in the infected cell for a range of structures, e.g., inclusion bodies, X bodies, and intracellular crystals, have been described and some have been used diagnostically while some have been shown to contain virus. The proportion of the total virus that is present in these histologically demonstrable forms seems to be small, at least in infections with viruses like tobacco mosaic virus which multiply extensively in the host. Some virus is also attached to the structure of the infected host. Thus Smadel & Wall (77) liberated vaccinia virus from the membranes of infected chick embryos by digestion with trypsin and Gard (14) found that this treatment increased the infectivity of extracts from the brains of mice suffering from poliomyelitis; he pointed out, however, that this did not of necessity mean that virus had been liberated. Similar results have been obtained with the residue left after the normal procedures used in the extraction of viruses from leaves. Tomato bushy stunt virus (78) when prepared by fine grinding or digestion with enzymes has the same properties as that prepared from sap but tobacco mosaic virus appears to be more highly aggregated in the sap (59). The interpretation of the results of fine grinding is complicated by the fact that mechanisms which grind sufficiently finely to liberate virus also bring about some destruction or firm anchoring to the fiber. This can be minimised by a preliminary incubation with commercial tryp-

sin. Similarly, the chloroplast fraction of leaves becomes attached to paper pulp when ground with it in the same way (79). The most efficient agent for liberating virus from these leaf residues that has so far been investigated is the mixture of carbohydrases present in the digestive fluid of the snail (80). More tobacco mosaic virus can be liberated by exhaustive digestion than was present in the original leaf sap and this bound virus may account for a third of the insoluble nitrogen of the leaf.

These phenomena show that virus occurs attached to the structural elements of the infected cell but they do not show whether this is the site of synthesis or whether the virus becomes fixed there after circulating in solution. It is also not clear whether the liberation is due to a mechanical dispersion of the cell structure or to the more specific destruction of a receptor to which the virus is fixed. There is abundant evidence that viruses do become fixed to susceptible and other cells and examples of this fixation with influenza virus have already been discussed (17, 43). Fixation can conveniently be studied with bacterial viruses and Pirie (81) has found that in *Bacillus megatherium* it depends on the presence of a substance, presumably a polysaccharide, which can be hydrolysed by lysozyme from egg white. Lysozyme released virus from heat-killed bacteria, and bacteria that had been incubated with lysozyme could no longer adsorb virus. Similar phenomena have not been looked for with other organisms but Anderson (82), by sonic disintegration of a virus attacking *Escherischia coli,* has made an enzyme solution that can lyse the bacterium if it has been killed by exposure to ultraviolet light. Egg white lysozyme also will lyse these cells but the virus enzyme will not lyse *Micrococcus lysodeikticus.* This enzyme is therefore similar to lysozyme but not identical with it; it is greatly to be hoped that the nature of its substrate will soon be ascertained. In this system the adsorbtion of the virus on the bacterium can only take place in the presence of *l*-tryptophane (83) or of some other amino acids and tryptophane derivatives (84) or of material liberated in the course of lysis. The mode of action of these substances is obscure and their presence at the time of lysis is necessary even with killed cells. Preliminary exposure of the virus to tryptophane is inefficacious; the effect, therefore, is not an activation or reactivation comparable to that brought about by amino acids on another bacterial virus after inactivation by aldehydes or aldoses (85).

General Methods

Ultracentrifugation plays such an important part in the separation of all but the most stable viruses that examinations of the technique are of great importance. Pickles (86) has shown both experimentally and by a theoretical discussion how the circulation of fluid in an angle centrifuge, caused by the descent of concentrated solution on the outside and the ascent of more dilute solution on the inside, affects both the rate of sedimentation and the formation of a boundary. By imposing a density gradient on the fluid in the centrifuge tube, through having a gradation in the concentration of an unsedimentable solute, this convective remixing can be avoided, for the gradient minimises the bulk movement of fluid around the tube. Successful use of this technique has been made in the study of influenza virus (19). The Sharples centrifuge, which was at one time much used in the separation of viruses, has to some extent been superseded by high speed centrifuges that carry the fluid in celluloid tubes. The Sharples is however still a more widely distributed type of centrifuge, and Markham (87) has discussed the conditions under which it can profitably be used to separate viruses from solution rather than simply to clarify the solution.

Much effort has been expended on the measurement of the dosage of x- or gamma-radiation needed to inactivate a certain proportion of a virus preparation because it was assumed that from this value the volume of the virus could be calculated (cf. 57). Friedewald & Anderson challenge this assumption (88) for they find that the dosage needed to inactivate rabbit papilloma virus increases with the virus concentration and that it is greatly increased by the presence of extraneous protein. It is argued that x-rays activate water molecules and that these inactivate the virus if they diffuse into it before acting on the extraneous matter or undergoing spontaneous deactivation. Further evidence for the indirect action of the radiation comes from an examination of the inactivation of tobacco mosaic virus (89). The inactivation caused by a given dosage is independent of the virus concentration if this is large or very small but there is a hundredfold difference between the two values. It is argued that the value for strong solutions or dry preparations can give a measure of the particle size. The uncertainties in this method are great, but it is a method that gives directly a value for the volume of the particle in the state

in which it exists in infective fluids. All other methods of determining the mass or volume of particles in this size range involve comparable uncertainties and they have the added disadvantage that they may be applicable only to purified preparations or to preparations that have been dried. It is obvious that some viruses are bigger than others, but, until better methods of measurements are available, the publication of tables setting out the sizes of all the viruses, sometimes to two or even three significant figures, is to be deprecated.

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ROTHAMSTED EXPERIMENTAL STATION
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INACTIVATION AND DETOXICATION OF PRESSOR AMINES

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The relationship between chemical structure and physiological activity is shown at its best in the pressor amines, the compounds related to epinephrine and ephedrine. In this group, which has a common parent in the β-phenylethylamine skeleton, it becomes possible to correlate almost vectorially the effect of substituents in the parent molecule with modification in pharmacodynamic properties, the effect of two or more substituents becoming more or less additive. These relationships have recently been reviewed by Hartung (1), by Graham, Cartland & Woodruff (2) and by Rajagopalan (3). The complete understanding of this correlation will have to wait, however, until the protoplasmic reaction of each member is established. Such biological reactions are often complex, involved, and elusive. Nevertheless, in many instances the ultimate fate of at least part of these compounds in the living organism is known; and from accumulated information about the biochemical behavior of the drug molecule some insight is being gained about the sequence of degradation reactions which the molecule undergoes in the tissues.

The aim of this review is to correlate the known data on the degradation, metabolism, detoxication, and elimination of pressor amines with their molecular structure. The degradations are enzymic; they will be considered under *in vitro* reactions; and then the corresponding *in vivo* and other reactions will be discussed.

"In Vitro" Reactions

Monoamine oxidase system.—Hare showed that the liver of most animals is able to effect oxidative deamination of tyramine, the amount of oxygen taken up depending on the conditions, but always bearing a stoichiometric ratio to the amine (4). It was recognized that a special system is involved in the uptake of the first atom of oxygen and that the immediate products are ammonia and an aldehyde (5). The responsible enzyme was shown to differ from α-amino acid oxidase (6), and that which had been called by such varied names as tyramine oxidase, epinephrine oxidase, and aliphatic amine oxidase was probably a single enzyme, and for it the term amine oxi-

dase was proposed (7, 8). In order to avoid possible confusion with diamine oxidase, it is suggested that the term monoamine oxidase be employed (9).

The deamination of primary carbinamines, that is compounds in which the amino group is attached to a primary carbon atom or —CH_2— group, proceeds in neutral or slightly acid solutions according to the general equations (8, 10, 11):

$$R-CH_2-\overset{+}{N}H_3 + O_2 \rightarrow R-CH=\overset{+}{N}H_2 + H_2O_2$$

$$R-CH=\overset{+}{N}H_2 + H_2O \rightarrow R-CH=O + \overset{+}{N}H_4$$
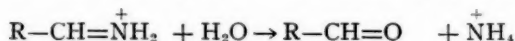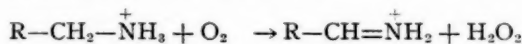
This mechanism resembles very closely the action of amino acid oxidase on α-amino acids (12). The rate of deamination is a function of R. The ammonia may be estimated by employing the Warburg technique (13, 14, 15); the aldehyde, unless fixed, e.g., as a hydrazone or bisulfite addition product (11), may undergo a Cannizzaro reaction, promoted by aldehyde mutase (6, 16, 17), to form the corresponding acid and alcohol. But in the presence of aldehyde fixatives the yield of aldehyde approaches the theoretical value (8).

An analogous deamination occurs with secondary methylamines, R — CH_2 — NH — CH_3, including epinephrine (8), with the formation of methylamine instead of ammonia. Tertiary amines, R — CH_2 — $N(CH_3)_2$, e.g., hordenine, also react to form an aldehyde and dimethylamine, but at a slower rate. Relative rates for deamination of typical amines have been determined by Alles & Heegaard (18), whose results serve to show, as Jacobsen & Gad (19) suspected, that it is perhaps a matter of related enzymes rather than of a single enzyme; their results also emphasize why it may frequently be difficult to reconcile data from different laboratories and why it is doubtful whether more than a qualitative correlation between the structure of the substrate and enzyme activity is possible.

The aliphatic normal primary amines are deaminated, beginning slowly with ethylamine (13) and reaching a high rate for the higher homologues (18). Branched chain compounds of structure RR′CH — CH_2 — NH_2 react more slowly than their straight-chain isomers. Secondary carbinamines, including 2-aminoheptane, RR′CH — NH_2, are completely unreactive (13, 18, 20). Likewise diamines with each of the amino groups attached to a primary carbon atom, NH_2 — $(CH_2)_n$ — NH_2, where n varies from 2 to 8, are unreactive (18).

The presence of a methyl group in the amino nitrogen atom, $R - CH_2 - NH - CH_3$, as a rule reduces the activity of the enzyme to approximately one half (18), although occasionally it may increase the rate (21). A second methyl group, as in $R - CH_2 - N(CH_3)_2$, reduces the activity still more or abolishes it altogether. Some activity may be displayed by the enzyme toward ethylamines of the type $R - CH_2 - NH - C_2H_5$ (13, 18).

Secondary carbinamines, as already mentioned, are refractory to monoamine oxidase. Thus isopropylamine and *sec.* butylamine are unaffected (13). Of three isomeric phenylpropylamines, $C_6H_5 - CH(CH_3) - CH_2 - NH_2$ and $C_6H_5 - CH_2 - CH_2 - CH_2 - NH_2$ undergo deamination, whereas $C_6H_5 - CH_2 - CH(CH_3) - NH_2$ is stable (13, 15, 20). The manner in which 1-phenyl-3-aminopropane, less toxic than amphetamine, is detoxicated does not yet appear (22) although it is affected by monoamine oxidase (18). Phenylethanolamine, $C_6H_5 - CHOH - CH_2 - NH_2$, is deaminated, but ephedrine, $C_6H_5 - CHOH - CH(CH_3) - NHCH_3$, is resistant (13). Epinephrine, $(HO)_2C_6H_3 - CHOH - CH_2 - NHCH_3$, is deaminated, but not so the isomeric cobefrine $(HO)_2C_6H_3 - CHOH - CH(CH_3) - NH_2$ (13, 23).

Not only are secondary carbinamines stable toward the monoamine oxidase system, but their presence inhibits the normal formation of ammonia in isolated tissues and inhibits respiration in brain cortex slices (11, 24). The inhibitory activity varies, depending on the amine and on the substrate (25); *sec.* amylamine and amphetamine show pronounced inhibition, ephedrine less. This phenomenon is attributed to the competition of the refractory amine with the active substrate for adsorption on the enzyme (11, 13, 26, 27).

Amines combine with the enzyme in a strictly reversible manner. The approximate dissociation constants of several inhibitory amines with the enzyme have been measured. The Ks values are: for isoamylamine 8×10^{-4} (18) and for amphetamine 4×10^{-4} (27).

The inhibitory action of amphetamine is also seen in the inhibition *in vitro* of glucose oxidation by brain tissue due to the presence of tyramine or other amines; and the inhibitory effect is observed in dilutions which approach pharmacological significance *in vivo* (27).

Phenolase and cytochrome systems.—Neuberg (28) in 1908 described a phenolase which inactivates epinephrine, and in 1933 Heard & Raper (29) showed that catechol oxidase oxidizes epinephrine. However, except for a number of arthropods, the amount of catechol

oxidase normally present in animal tissues is comparatively low and plays no significant role in the biological inactivation of epinephrine (30), unless it later may develop that there is involved a group of closely related enzymes of which some members are very specific (31, 32) and some of which may, perhaps, be found in those tissues which are inhibited by epinephrine (33, 33a).

Colorimetric assays in which epinephrine is autoxidized or treated with oxidizing agents have long been known (34, 35), but little was known of the structure of the products until Richter & Blaschka (36) identified a product which they called iodoadrenochrome; it was obtained by treating epinephrine in dilute acetic acid with potassium iodate, according to the equation:

$$C_9H_{13}O_3N \cdot CH_3 \cdot COOH + KIO_3 \rightarrow C_9H_8O_3NI + CH_3 \cdot COOK + 3H_2O$$

Iodoadrenochrome has been identified as 1-methyl-2-iodo-3-hydroxy-2,3-dihydroindole-5,6-quinone.

Epinephrine, oxidized at pH 5 with catechol oxidase, takes up approximately one molecule of oxygen per mole; analysis of the unstable product suggests a hemihydrate of formula $C_9H_9O_3N \cdot \frac{1}{2}H_2O$; treatment with iodine forms iodoadrenochrome. These reactions proceed according to the equation:

| Epinephrine | o-Quinone derivative | Adreno-chrome | Iodoadrenochrome |

Adrenochrome is quite unstable, especially in more alkaline solutions, readily forming melanin pigments.

Epinephrine, as well as flavin and flavoprotein, in the presence of coenzyme I, is capable of acting as a carrier in the complex oxidation mechanism of the malic or lactic acid dehydrogenase systems (37). The observed turnover number is 5, which compares favorably with that for flavoprotein and represents high catalytic activity. There is a lag before oxidation sets in, undoubtedly until adrenochrome has first formed, for this pigment acts in the same manner as the carrier but without the lag that is observed with epinephrine. Equally as effective as epinephrine in this system are (+)epinephrine, epinine,

cobefrine, and its N-methyl derivative. The specificity of the substrate is complete, however, only $l(+)$-lactic and $l(-)$-malic acids being oxidized. The proposed scheme for the reaction is:

I. O_2	+ reduced cytochrome	→ oxidized cytochrome	+ H_2O_2
II. Oxidized cytochrome	+ epinephrine	→ reduced cytochrome	+ adrenochrome
III. Adrenochrome	+ reduced coenzyme	→ leuco-adrenochrome	+ oxidized coenzyme
IV. Leuco-adrenochrome	+ O_2	→ adrenochrome	+ H_2O_2
V. Oxidized coenzyme	+ lactate	→ reduced coenzyme	+ pyruvate

Epinephrine and leucoadrenochrome are easily oxidized by the indophenol-cytochrome system.

Whether adrenochrome is the agent immediately responsible for the dehydrogenation of glyceraldehyde by epinephrine (38) has not been established, but it is suggested that for this reaction epinephrine acts as a specific hydrogen-transport catalyst.

The dehydrogenation of succinic acid to fumaric acid and maleic acid by epinephrine (39, 40) undoubtedly depends first on the formation of an o-quinone which, like o-benzoquinone (41), may act as a hydrogen transporter; or perhaps it depends on adrenochrome.

The first step in the oxidation of epinephrine is accompanied by a change in color and loss of pressor activity, and probably involves the formation of the o-quinone derivative (42, 43); in the second step the color changes to yellow-brown (43), and the ring closure is analogous to a similar reaction for dihydroxyphenylalanine (DOPA) (44).

If the enzymic oxidation is carried out, for instance, in the presence of the nongravid cat uterus, or with stomach extract, in neutral solution and with epinephrine at 1×10^{-6} to 1×10^{-5} M, the formation of the colored adrenochrome is observed during the first fifteen minutes, during which period it loses its hypertensive properties; suddenly, after forty to ninety minutes, the color pales, and the solution now takes on hypotensive properties (33). Further oxidation causes precipitation of melanin. The hypotensor formed in the second stage has been called adrenoxine by Bacq & Heirman, its discoverers, and quite extensively investigated by them (45 to 53); however, its

isolation in pure form has not yet been reported. It may be obtained from the isomeric epinephrines (47) and perhaps also from hydroxytyramine (54). The responsible agent is probably not a phenolase (43, 55).

The effect of various substances on the oxidation of epinephrine by tyrosinase was studied by Martin and co-workers (56). A definite structural relationship was found in those substances inhibiting oxidation; thus aniline, benzoic acid, and the three isomeric aminobenzoic acids show inhibitory action. Aminophenol and the hydroxybenzoic acids accelerate the oxidation.

These enzyme systems also react on monohydroxy phenols. Isolated from potatoes was a phenolase which activates compounds having one or two phenolic groups (15). A hydroxyl group in the side chain of a pressor amine retards the rate of oxidation (15).

The oxidation of tyramine by tyrosinase, as worked out by Dulière & Raper, proceeds as follows:

Tyramine   3,4-Dihydroxy   o-Quinone
                        phenylethylamine   derivative

Melanin ←   Dihydroxy
                   indole intermediate

N-methyltyrosine is oxidized at approximately the same rate and ultimately also forms pigment; presumably it follows the same route and goes through the N-methylindole intermediate. The corresponding tertiary amine, and the quaternary ammonium salt, although taking up appreciable amounts of oxygen, do not form pigments and would not be expected to form indole intermediates unless there is first an unexpected demethylation; they must therefore be oxidized by an alternative route (58). Hydroxytyramine and its N-methyl derivative take up oxygen at about half the rate of tyramine (58). The isomeric 2,3-dihydroxyphenethylamine takes up oxygen appreciably slower than hydroxytyramine.

1-p-Hydroxyphenyl-2-aminopropane, its N-methylderivative, and p-hydroxyephedrine under ideal conditions take up five atoms oxygen per molecule: *in vitro* from 15 to 40 per cent of the amine can be accounted for by the ammonia liberated. To account for these results Beyer (15) offers the following mechanism:

A   (HO—phenyl)CH$_2 \cdot$CH(CH$_3$)$\cdot$NH$_2$ $\xrightarrow{\frac{1}{2}O_2}$ (HO, HO—phenyl)CH$_2 \cdot$CH(CH$_3$)$\cdot$NH$_2$

B   (HO, HO—phenyl)CH$_2 \cdot$CH(CH$_3$)$\cdot$NH$_2$ $\xrightarrow{O_2}$ (O=, O=—ring)CH$_2 \cdot$CH(CH$_3$)$\cdot$NH$_2$ + H$_2$O$_2$

C   (O=, O=—ring)CH$_2 \cdot$CH(CH$_3$)$\cdot$NH$_2$ $\longrightarrow$ (HO, HO—phenyl)CH$_2 \cdot$C(CH$_3$):NH

D   (HO, HO—phenyl)CH$_2 \cdot$C(CH$_3$):NH $\xrightarrow{H_2O}$ (HO, HO—phenyl)CH$_2 \cdot$CO$\cdot$CH$_3$ + NH$_3$

E   (HO, HO—phenyl)CH$_2 \cdot$CO$\cdot$CH$_3$ $\xrightarrow{\frac{1}{2}O_2}$ (O=, O=—ring)CH$_2 \cdot$CO$\cdot$CH$_3$ + H$_2$O

Step (A) is analogous to the formation of DOPA from tyrosine (59, 60) and of catechol from phenol (61); the formation of hydrogen peroxide is debatable (62). Further oxidation of catechol derivatives to quinones of related structure, step (B), is well established (42, 63, 64); the hydrogen peroxide formed during this oxidation probably promotes the enzymic reaction (65, 66). Step (C), as further illustrated by the second set of reactions below, is essential in oxidizing the amino group; after hydrolysis of the imino group, step (D), the diphenolic ketone is oxidized by the fifth atom of oxygen to the corresponding quinone, which, in turn, promotes the deamination in step (C). Support for this mechanism is found in the oxidation of 1-(3,4-dihydroxyphenyl)-2-aminopropane, in which the second phenolic hydroxyl group is already present in the 3-position; it takes up but three atoms oxygen per molecule of substrate.

Step (C), in which the quinone is reduced to the dihydric phenol and the amino group oxidized to the imino, is probably not intramolecular as indicated. It is similar to the oxidative deamination of glycine by phenol oxidases in the presence of phenol, p-cresol, or

4-methyl-1,2-quinone (63, 67, 68). Beyer (69), allowing phenolase to react on amphetamine in the presence of p-cresol, obtained evidence for at least partial deamination, the ammonia recovered varying from 11 to 55 per cent of the amine, depending on the concentration of amphetamine and the p-cresol. Deamination may well have proceeded farther than the recovery of ammonia indicated, since there is a possibility of side reactions that would not permit quantitative isolation. The mechanism proposed for the liberation of ammonia is:

$$C_6H_5 \cdot CH_2 \cdot C(CH_3):NH \xrightarrow{H_2O} C_6H_5 \cdot CH_2 \cdot CO \cdot CH_3 + NH_3$$
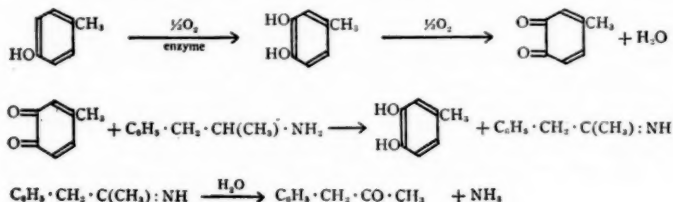
It will be observed that the second step is very similar to step (C) in the preceding series of reactions.

The oxidation rates for primary amines and for secondary methylamines, e.g., for tyramine and N-methyltyramine, are nearly the same; hordenine, the corresponding tertiary amine, reacts at a much slower rate (70). The presence of an alcoholic hydroxyl on the phenyl-bearing carbon atom in pressor amines retards considerably the rate at which oxidation proceeds; and if the secondary alcohol is oxidized to a ketone, practically no oxidation takes place. The dipole moment for the alcoholic hydroxyl group is -1.83×10^{-18} e.s.u. and for the ketonic oxygen atom -2.79×10^{-18} e.s.u.; their presence in the molecule reduces the reactivity of the p-hydroxy-substituted aromatic nucleus (70). This retardation or inhibition is effective only for the first step of the oxidation, for once the catechol structures have been formed the rates of oxidation, e.g., for epinephrine and its corresponding ketone, show no appreciable difference, even the ketonic derivative being oxidized.

Compounds with a single phenolic hydroxyl group in the m- or o-position are reported to be unaffected by the enzyme (71, 72); m-hydroxyphenethylamine, however, may be slowly oxidized by tyrosinase (58).

The ratio of cresolase and catecholase activities for different mushroom preparations is not greatly altered by purification (72). The

dissociation constants for the enzyme-amine combinations are of the same order of magnitude, varying from 2.2×10^{-3} for tyramine to 2.7×10^{-3} for *dl*-ephedrine (72).

The immediate oxidation products of a phenolic pressor amine by cytochrome-*c* or by a phenolase inhibit the activity of the monoamine oxidase system (73).

Ascorbic-dehydroascorbic acid system.—It is known that ascorbic acid will deaminate certain amino acids (74, 75, 76), iminazoles (77, 78), and purines. Beyer (69, 71) observed that a similar deamination is effected with amphetamine. The reaction, starting with dehydroascorbic acid, may be written:

$$\begin{array}{c} \text{OC} \\ \text{OC} \\ \text{OC} \quad \text{O} \\ \text{HC} \\ \text{HOCH} \\ \text{CH}_2\text{OH} \end{array} + \text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CH(CH}_3) \cdot \text{NH}_2 \longrightarrow \begin{array}{c} \text{OC} \\ \text{HOC} \\ \text{HOC} \quad \text{O} \\ \text{HC} \\ \text{HOCH} \\ \text{CH}_2\text{OH} \end{array} + \text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CH(CH}_3) : \text{NH}$$
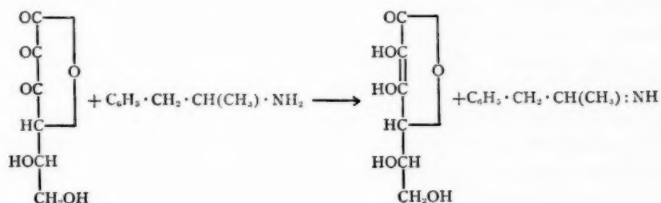
It is likely, however, that the actual reaction is more complex, for as it proceeds the mixture assumes a golden-yellow color; and it is known that ascorbic acid is oxidized in the presence of primary amines and in alkaline medium to give rise to a series of oxamides and N-substituted analogues (79). Employing *in vitro* experiments, Beyer (69) was able to recover ammonia equivalent to 27.6 to 55.5 per cent of the amphetamine; the formation of the ketone was established by its isolation as the 2,4-dinitrophenylhydrazone (80).

The ascorbic-dehydroascorbic acid system, as distinguished from the monoamine oxidase system, is active on secondary carbinamines as well as on primary carbinamines, but like monoamine oxidase, is about as effective on secondary amines as on primary amines. Thus ephedrine produces methylamine about equivalent to the ammonia obtained from propadrine. The tertiary amine N,N-dimethylamphetamine is resistant to deamination.

The introduction of the alcoholic hydroxyl into the amphetamine molecule decreases markedly the deamination, from 31 to 54 per cent for amphetamine to 9 to 12 per cent for propadrine (80).

Phenolic pressor amines are not deaminated by the ascorbic acid system, but under the conditions of reaction there is color formation,

the rate of its development depending on the amine. The reactions are represented as follows (80, 81):

$$\left[\begin{array}{c}\text{tyramine}\\ +\\ \text{ascorbic}\\ \text{acid}\end{array}\right]\xrightarrow{O_2}\left[\begin{array}{c}\text{hydroxy-}\\ \text{tyramine}\\ +\\ \text{dehydro-}\\ \text{ascorbic}\\ \text{acid}\end{array}\right]\rightleftharpoons\left[\begin{array}{c}o\text{-quinone}\\ \text{derivative}\\ \text{of tyramine}\\ +\\ \text{ascorbic}\\ \text{acid}\end{array}\right]$$
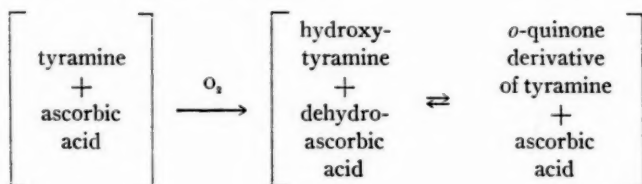
The test for the o-dihydroxyphenyl nucleus, as developed by Richter (82), was positive, and the color developed slowly or not at all if the phenol or the vitamin were omitted from the reacting system.

Other systems.—Toscano Rico & Malafaya (83) observed that epinephrine is inactivated *in vitro* by the presence of acetaldehyde, pyruvaldehyde, or glyceraldehyde. This inactivation was studied in detail by Bayer & Wense (84). They found that acetaldehyde, usually looked upon as a strong reducing agent, promotes the oxidation of epinephrine. For example, a solution of epinephrine containing acetaldehyde maintained at incubator temperature in the absence of oxygen showed no loss in activity. Oxygen bubbled for five minutes through a solution of epinephrine alone caused no appreciable loss of activity; but oxygen bubbled for five minutes through a solution of epinephrine containing acetaldehyde produced deterioration in activity.

A similar result was observed with tyramine. Oxygen alone, even after several hours, had no effect, but in the presence of aldehyde there was pronounced loss of activity.

Acetaldehyde alone does not bind epinephrine; neither does glyceric aldehyde nor acetone. Formaldehyde, however, reacts to form a compound to which is assigned the structure $[(HO)_2C_6H_3 \cdot CHOH \cdot CH_2 \cdot N(CH_3) \cdot]_2CH_2$. Proteins, in the presence of lactic acid, bind epinephrine with considerable intensity (85). However, the biological significance of these reactions appears not to be established.

"IN VIVO" REACTIONS

The question of how closely the results of controlled *in vitro* experiments parallel actual reactions *in vivo* is not easily answered, since the possibility of greater complexity and the influences of in-

hibitors and competing reactions are always present in the intact organism. Nevertheless, many clues are available which throw considerable light on the *in vivo* mechanisms which inactivate the pressor amines.

Primary carbinamines.—The monoamine oxidase system, which degrades amines of types $R \cdot CH_2 \cdot NH_2$ and $R \cdot CH_2 \cdot NHCH_3$ *in vitro,* is effective *in vivo* only on proteinogenous and related amines. Thus Ewins & Laidlaw (86), who fed tyramine to dogs, were able to isolate from the urine *p*-hydroxyphenylacetic acid equivalent to 40 per cent of the amine. Guggenheim & Löffler (87) isolated not only the acid but also tyrosol, $p\text{-}HOC_6H_4 \cdot CH_2 \cdot CH_2OH$. They also observed the rapid detoxication of many other amines, and in several instances they isolated the corresponding acid and alcohol, products to be expected from the action of aldehyde mutase acting on the aldehyde formed from the primary carbinamine.

Monoamine oxidase is widely distributed, being found in mammalian intestine, kidney, brain, lung, and especially in the liver (88). It is undoubtedly the agent which inactivates the amines studied by Richter (89). Following the administration of isoamylamine (100 mg.), benzylamine (160 mg.), phenylethanolamine (50 mg.), and phenethylamine (300 mg.) no increase in excretion was detected. Following phenethylamine, phenylacetic acid equivalent to 40 per cent of the amine was found in the urine during the following 2.5 hours, and 62 per cent during 4.5 hours. This suggests rapid degradation.

The rise in blood pressure produced by intravenous phenethylamine persists for only several minutes. Richter, Lee & Hill (90) administered 75 mg. to a patient weighing 62 kg.; after 2.75 minutes the blood pressure was back to normal. If such observations were to be taken as the sole criterion of inactivation, this would suggest a rate equivalent to about 26 mg. per kg. per hr. In order to establish more reliably the rate of inactivation, these investigators (90) determined the rate of disappearance of isoamylamine from the blood stream. Patients receiving 500 mg. showed complete removal in two to eight minutes, equivalent to 60 to 250 mg. per kg. per hr. Blood removed from one patient 100 seconds after administration of the amine had a strong odor of isoamyl alcohol. Perhaps the rapid disappearance of the amine from the blood stream is due to diffusion into the tissues, but the final elimination must be caused by irreversible detoxication.

Such inactivation is of interest not only because of possible in-

toxication by amines formed by the putrefactive decarboxylation of α-amino acids (90) but for possible use in experimental hypertension.

Experimental hypertension is believed by some to be due to the liberation into the blood stream of substances formed in the ischemic kidney (91), and it is postulated that these substances may be amines (54, 92, 93). Oster & Soloway (91) found that the intramuscular injection of amine oxidase into experimentally hypertensive rats produced no significant lowering of blood pressure; but since the ischemic kidney suffers from depletion of oxygen, they felt it might be lack of oxygen rather than inactivity of the enzyme which is responsible. Since Philpot (16) reported that o-cresolindophenol acts as a hydrogen acceptor from amines in the presence of amine oxidase, these investigators examined the possibility of destroying with this agent any hypertensive amines that might be formed in the kidney; intramuscular injection of 10 mg. o-cresolindophenol into hypertensive animals caused a drop of 30 to 40 mm. in the blood pressure in three out of six rats; the blood pressure of six controls was not affected by a dose of the same size.

Tyrosinase reduces the blood pressure of perinephritic hypertensive dogs if catechol be given simultaneously (56).

Bernheim & Bernheim (94) believe that inactivation of phenolic compounds may be more complex than simple deamination. Kidney and liver slices are able only to deaminate tyramine; but heart, skeletal, and possibly smooth muscle of the rat are able not only to deaminate tyramine but also to destroy simultaneously the phenolic hydroxy groups, presumably by breaking down the aromatic nucleus.

Mescaline, on which monoamine oxidase is slowly active *in vitro* (11, 13, 14), is tolerated by the rabbit in doses many times greater than that which is toxic to man. In the rabbit it appears to be detoxicated by amine oxidase and a cyanide-sensitive factor (95). Dogs and rabbits degrade mescaline to 3,4,5-trimethoxybenzoic acid (14, 96). In man mescaline is excreted unchanged, at least in part, in the urine (89).

The possibility of inactivating primary amines *in vivo* by means of aldehydes, especially the higher aldehydes or "plasmal" of Feulgen (97), by the formation of Schiff bases, is raised by Oster and co-workers (93, 98).

Secondary carbinamines.—Richter (89) administered ephedrine, desoxyephedrine, and amphetamine, both orally and intravenously, to normal male adults. In every instance he found unchanged and

unconjugated amine in the urine. For ephedrine the maximal excretion was 111 μg. per ml. after four hours; a subject receiving 104 mg. excreted 77 per cent in twenty-four hours and was still excreting at the rate of about 0.25 mg. per hr. after thirty-six hours. Amphetamine was excreted more slowly; a subject who received orally 14.7 mg. of the base excreted 40 per cent in the first twenty-four hours and 70 per cent in forty-eight hours. Several hours after administration, the concentration of amphetamine in the urine was 10 to 13 μg. per ml.; thus, with a concentration at the most of 2.1 μg. per ml. in the blood, this shows that the kidneys have the power to effect considerable concentration. Desoxyephedrine is also excreted slowly.

On the basis of his results Richter was led to believe that these amines are eliminated quantitatively in the urine. However, Beyer & Skinner (99), working with larger numbers of subjects, were able to show that such is not the case. They administered amphetamine sulfate orally in doses of 10, 20, or 30 mg. to fifteen subjects and were able to collect from 22.1 to 66.3 per cent, average 43 per cent, of unchanged amine during forty-eight hours. They observed maximum excretion during three to twelve hours after ingestion; after twelve to eighteen hours a decrease in excretion was frequently observed. The amount of drug excreted varied with the corresponding urine volume, but decreased progressively. That incomplete excretion is not due to incomplete absorption from the intestine is shown by the fact that dogs receiving orally 10 mg. amphetamine sulfate excreted as much or more than when the same animals received the same size dose parenterally. Furthermore, if the liver function of dogs was impaired with carbon tetrachloride before administering the amine, the concentration of the amphetamine in the urine increased and was quantitatively eliminated in twenty-four hours. The period for total excretion of amphetamine by animals with pathological livers approaches the time over which the pharmacological effects of the drug are observable. The fact that in normal humans and dogs the period of excretion so markedly outlasts the physiological effects suggests that there must be some intermediary mechanism, comparatively slow though it may be, for inactivating the drug until it can be eliminated. Perhaps it forms with some agent in the body a loose combination from which it may be reversibly released.

Jacobsen & Gad (19) attempted to follow the fate of amphetamine after injection into the ear vein of the rabbit. No amine was found in

the respired air. Within five minutes the concentration of the amine in the kidney was up to seven fold the concentration in the blood and maintained that approximate ratio for six hours. The amine appeared in relatively high concentration in the central nervous system. Some appeared in the stomach from which it was reabsorbed. The concentration in the liver was lower than in the kidney, probably because this is the chief site of degradation. The liver is capable of degrading one third of a dose of 600 mg. in six hours (100). This high rate of destruction no doubt explains why, in the rabbit, it was possible to recover from the urine only 10 per cent of an administered dose of amphetamine (19).

The inactivation of that part not excreted probably depends on the ascorbic-dehydroascorbic acid system. This is indicated by the finding that if ascorbic acid is fed to animals receiving amphetamine, the amount of amine appearing in the urine is decreased. The normal excretion by dogs receiving daily doses of 5 mg. amphetamine sulfate was reduced by about 35 per cent below the controls when they were fed 200 to 400 mg. ascorbic acid simultaneously; as the level of ascorbic acid was reduced, the ratio of excreted amine rose to former levels (69).

Although (+)amphetamine is more active pharmacologically than the (−)isomer (101), the difference in the rates of excretion of the two enantiomorphs seems to depend more on the subject than on the compound (99).

Ephedrine and propadrine are presumably inactivated, as is amphetamine (15). Yet the mechanism by which these compounds produce their circulatory effect is not clear. Gaddum (102) postulated that they protect, or inhibit, the *in vivo* destruction of the substance or substances liberated by the adrenergic nerves. This hypothesis is based on the observation that the addition of ephedrine, even as low as 1×10^{-5} M, to a solution of epinephrine perfused through a rabbit's ear greatly increased the effect of the epinephrine, although ephedrine by itself at 1×10^{-3} M was without effect. This action of ephedrine is similar to that produced by stimulating the adrenergic nerve. Or, when ephedrine was perfused through the ear, the ephedrine itself producing no vasoconstriction, the perfusate gave positive tests for what was strongly suspected of being epinephrine (103). Thus ephedrine may be thought of as acting on the circulation by virtue of either (a) the protection it affords the stimulating agent, i.e., epinephrine, as it is normally liberated at the nerve endings, is

protected by ephedrine from the usual epinephrine antagonists, or
(b) by stimulating secretion of the agent (104).

Whether ephedrine acts in the tissue by inhibiting the monoamine
oxidase system as Gaddum supposed, is open to question, especially
in view of later indications that this system probably does not func-
tion in the biological inactivation of epinephrine as was once assumed.

On the other hand evidence clearly suggests that the activity of
the nonphenolic secondary carbinamines on the circulation after oral
administration (105) is due perhaps to the low concentration of
phenolase in the tissues and to the refractivity of these compounds
toward monoamine oxidase. The oral activity of cobefrine (106, 107)
is less readily accounted for, especially since it appears to be inacti-
vated by a mechanism that is discussed later. But the explanation
for the complete inactivity of phenylbutanolamine, $C_6H_5 \cdot CHOH \cdot$
$CH(NH_2) \cdot CH_2 \cdot CH_3$, and its higher homologues on the blood
pressure (108) does not appear in the information thus far available.

Epinephrine, epinine, and cobefrine.—Epinephrine, whose natural
synthesis by the decarboxylation of the corresponding amino acid is
doubtful (54, 55, 109 to 112), although serving as a satisfactory sub-
strate *in vitro,* is probably little affected, if at all, by monoamine oxi-
dase *in vivo.* Judged by the duration in the rise of blood pressure
epinephrine appears to be rapidly inactivated. It can be destroyed in
the liver of the intact animal (113). Injected into the splenic vein
epinephrine is carried directly to the portal system and produces
about a fourth the rise in blood pressure that would be expected of
the same dose injected into the jugular vein. The simultaneous in-
jection of methylene blue, which is known to inhibit the amine oxidase
in the liver, into the splenic vein increases the effect of the epinephine;
o-cresolindophenol, which does not inhibit the enzyme, has no such
effect. Although it would appear that the liver may be the chief site
of inactivation for epinephrine, the precise role of monoamine oxidase
is difficult to establish, for in animals from which the liver function
has been excluded by evisceration the effect of methylene blue is not
clear cut (113).

From a study of the quantitative destruction of epinephrine by
liver enzyme *in vitro* Richter & Tingey (114) concluded that mono-
amine oxidase reacts too slowly to account for the rapid inactivation
observed. They estimated, for example, that the cat liver can inacti-
vate in about twelve minutes approximately half the epinephrine
present at a concentration of $1 \times 10^{-7} M$ (a physiological concentra-

tion). Hence, unless the monoamine oxidase system is more active in the tissues than in the test tube, it alone can hardly account for the rapid removal of the injected hormone from the circulation, although it is not impossible that inactivation may be effected by a modified amine oxidase (115).

Inactivation of epinephrine by the phenolase systems seems unlikely because of the absence of catechol oxidase from mammalian tissues (30), unless it should develop, as Cadden & Dill (31) and Hogeboom & Adams (32) suggest, that the organism may contain some highly specific phenolase.

The cytochrome system is abundant and, as already discussed, is capable of destroying epinephrine *in vitro*. But the living organism also contains many agents, such as amino acids, ascorbic acid, and glutathione, which inhibit the action of these systems (116, 117). Then, too, Keilin & Mann (65) found that although cytochrome-*c* is able to oxidize quinol and catechol *in vitro*, these compounds escape such oxidation *in vivo* and are excreted in conjugated form. Therefore it is difficult to assay the part this enzyme system plays in detoxicating epinephrine.

Bacq & Heirman (45 to 53) are of the opinion that adrenoxine, formed in the tissues by the oxidation of epinephrine, or a closely related substance, plays an essential role in the biochemistry of the organism, and they regard it as significant that just those tissues on which epinephrine has an inhibitory effect are most capable of oxidizing it *in vitro*. Adrenoxine possesses a mydriatic action 10,000 times greater than epinephrine (118) and has cardioinhibitory properties (50). That it or a similar substance may be formed in the animal body is suggested by the findings of Raymond-Hamet (119) that epinephrine may cause vasodilatation by peripheral action after vasoconstriction; the epinephrine passing through the heart is oxidized (120). The formation of adrenoxine, or possibly of adrenochrome, is responsible for the inhibitory effect of epinephrine on pendular movements and tonus of rabbit intestine (121). In any case the formation of a depressor substance from epinephrine depends on the presence of the phenolic hydroxyl groups (122).

Several groups of investigators report that *o*-quinone derivatives of pressor amines act as "antipressors," that is, reduce an elevated blood pressure to normal (123). After oxidation by mushroom catecholase (124) or by the presence of heavy metal ions (125), epinephrine antagonizes the effect of pressor amines in the unan-

esthetized cat and lowers the blood pressure of rats and dogs made hypertensive by perinephritic scar. Iodoadrenochrome, administered parenterally, lowers the blood pressure of experimentally hypertensive rats without the accompaniment of toxic manifestations or fever; it does not affect the blood pressure of normal animals (123).

Since none of the enzyme systems known to be present accounts satisfactorily for the rapid inactivation of epinephrine (114), Richter approached the problem from a new angle (126); he tried to identify the products in the urine following epinephrine administration. If the amine oxidase is the chief agent, then protocatechuic acid, in conjugated form, should be present. If phenolase or cytochrome is the chief agent, then indole derivatives might be expected. To secure further evidence on the *in vivo* behavior of the different systems toward pressors containing the catechol nucleus, he included in his study epinine, cobefrine, and (+)epinephrine.

Richter took 50 mg. cobefrine by mouth, but with a sensitive test he could detect none of the free secondary carbinamine in the urine. After hydrolyzing the urine with sulfuric acid, however, he was able to adsorb on alumina a substance which gave characteristic tests for cobefrine. It was estimated that the excretion of the conjugated amine was maximal during the second to sixth hours and still appreciable at the ninth hour; the total amount excreted during the first nine hours was 24 mg., and elimination was incomplete at that time.

Epinine, toward which the monoamine oxidase system is very reactive (13, 18), might be expected to be degraded in the organism like phenethylamine, unless inactivation by conjugation is the more rapid process. Following its ingestion Richter could find no free epinine in the urine; after hydrolysis, but not before, characteristic color reactions for epinine were obtained with ferric chloride. No trace of protocatechuic acid could be found. The recovered epinine, estimated colorimetrically, was 30 to 60 per cent.

(+)Epinephrine likewise appeared in the urine as a conjugated derivative. After taking 55 mg. by mouth Richter recovered 39.2 mg. in twenty-one hours. The rate of elimination was maximal after four hours.

(−)Epinephrine is commonly stated to be inactive when taken orally (106), but this is open to question (126, 127). Because of the severe systemic reactions following its oral ingestion, Richter was obliged to confine himself to smaller doses. After taking 10 and 30 mg. on separate occasions, he was unable to detect free epinephrine,

protocatechuic acid, or an indole derivative in the urine. After hydrolysis, the presence of free epinephrine was confirmed by numerous tests, including pressor effects on spinal cats. Elimination of the epinephrine conjugate reached a maximum rate in about five hours; and in twenty-four hours 50 to 70 per cent of the original dose was recovered. After the dose of 30 mg. the concentration of epinephrine in the urine rose to 33 µg. per ml. after five hours; at that time the concentration in the blood could have been no higher than 6 µg. per ml., indicating that the kidneys must be able to concentrate to a high degree the conjugated product.

Since the color test with ferric chloride did not appear until after hydrolysis, conjugation was presumed to take place through a phenolic hydroxyl group. Tests for a glycuronide were negative. Tests for a sulfate ester, on the other hand, were positive. It is suggested, therefore, that the conjugate exists as a zwitterion of structure

$$-\overset{-}{O} \cdot SO_2 \cdot O \cdot C_6H_3(OH) \cdot CHOH \cdot CH_2 \cdot \overset{+}{N}H_2CH_3.$$
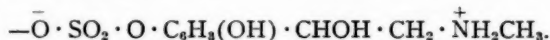
Further studies by Richter & MacIntosh confirmed the formation of sulfate ester (128).

Esterification with sulfuric acid may follow the same mechanism in the liver which detoxicates phenols by conjugation (65, 129). Indirect evidence is seen in the work of Torda (130), who found that cocaine inhibits the phenol sulfur esterases; in the results of Bernheim & Bernheim (131), who report that guinea pig liver catalyzes the *in vitro* conjugation of phenols; and in the observation of Deichmann (132) that the administration of epinephrine causes increased excretion of organic sulfates. Direct evidence is seen in the report of Loeper and co-workers (133) that tyramine esterified through its phenolic hydroxyl group with sulfuric acid loses its pressor properties; the conjugated epinephrine has no pressor properties (128). Or perhaps the inactivation depends on a special "sulfosynthase" system (128).

The excretion of catechol compounds as a conjugate which may be hydrolyzed to regenerate the original compound has been confirmed by Beyer & Shapiro (23). They found maximal excretion in dogs within the first four hours after administration. From the urine of human subjects receiving 30 mg. cobefrine hydrochloride by mouth, the drug recovered during twenty-four hours varied from 16.7 to 21.4 mg.; from men receiving 30 mg. epinine hydrochloride by mouth, the amounts recovered varied from 17.9 to 27.8 mg. The

amount of epinine recovered depended on the size of the dose and the route of administration, being larger after oral ingestion.

Simple esterification of a phenolic hydroxyl group with sulfuric acid does not require oxygen, yet liver slices do not inactivate epinephrine in the absence of oxygen (134). However, Arnolt & DeMeio (135) report that the *in vitro* conjugation of phenols by enzymatic processes, although the conjugation itself consumes no oxygen, requires the energy released by reactions in which oxygen is consumed.

In view of these results it appears that most of the epinephrine, as well as epinine and cobefrine, which is administered is inactivated by the formation of the sulfate. Whether this is true also for the hormone as it is elaborated by the suprarenal medulla still remains to be demonstrated. Epinephrine normally secreted may be inactivated by other mechanisms (136).

The potentiation of epinephrine by substances like cocaine (137) is attributed to the possibility that they interfere with inactivation processes (138).

The chemical structure of all these pressor compounds is known. In this review an effort has been made to correlate the mechanisms of their inactivation, detoxication, and elimination with structure. To establish more completely a correlation between chemical structure and pharmacodynamic properties it will be necessary to determine the biochemical processes through which the biological responses of these compounds are mediated.

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ERRATA

At Professor Neuberg's request the following corrections and alterations in his review (pp. 435 to 474) are brought to the attention of the reader:

Page 435, subhead: for MINERAL REQUIREMENTS read

MINERAL CONTENT AND REQUIREMENT

Page 435, line 12 from bottom: *for* uranous oxide *read* uranyl.

Page 435, line 6 from bottom: *for* Verzar *read* Verzár.

Page 437, line 4: *insert* per cent *after* 10^{-4} and 10^{-5}.

Page 438, line 21: *after* degradation *insert* (Buchner and Harden).

Page 438, line 14 from bottom: *for* has also stated *read* already took in consideration.

Page 438, line 12 from bottom: *for* immediate *read* rapid.

Page 438, last line: *after* diphosphate *insert* (Neuberg & Leibowitz).

Page 439, line 13: *for* Bromel *read* Brömel.

Page 439, line 5 from bottom and last line also: *for* arsenite *read* arsenate.

Page 440, line 14 from bottom: *for* Willstatter *read* Willstätter.

Page 441, mid page: *for* Teauloz *read* Jeanloz.

Page 441, line 17 from bottom: *delete sentence beginning* By oxidation.

Page 441, line 11 from bottom: *for* Garzully-Janke *read* Garzuly-Janke.

Page 442, line 9 from bottom: *for first formula in line read* $C_{30}H_{51}OCl$.

Page 443, line 18 from bottom: *for* Rimington (73, 74) has *read* Rimington (73) and Kench & Wilkinson (74) have.

Page 443, lines 5 and 6 from bottom: *for* sulfate *read* hydrosulfite.

Page 444, line 13: *for* components *read* components—.

Page 444, line 21: *for* of the *read* of.

Page 444, line 4 from bottom: *for* zylose *read* xylose.

Page 446, lines 5 and 6: *for* is fermented *read* is said to ferment.

Page 446, line 13 from bottom: *for* furanose *read* -furanose.

Page 446, line 6 from bottom: *for* also fermented in its furanose modification *read* fermented in both its modifications equally.

Page 447, line 6: *for* 5-*d*-fructonese *read* 5-*d*-fructonose.

Page 447, line 4 from bottom: *for* glycollicaldehyde *read* glycolaldehyde.

Page 447, line 2 from bottom: *for* glycollic *read* glycolic.

Page 448, line 2: *for* glycollicaldehyde *read* glycolaldehyde.

Page 448, line 19 from bottom: *for* objective *read* result.

Page 449, line 20: *for* Bakers' yeast *read* Some bakers' yeasts.

Page 449, line 13 from bottom: *for* $[C_6H_{10}O_4(PO_4H)]_2Ba$ *read* $C_6H_{10}O_4(PO_4H)_2Ba$.

Page 449, line 11 from bottom: *for* $-4.04°$ to $4.15°$ *read* $+4.04°$ to $+4.15°$.

Page 450, line 12: *for* galactopyranose-1-monophosphate *read* *d*-galactopyranose-1-monophosphate.

Page 450, line 22: *for* & *read* and.

Page 450, line 16 from bottom: *for* α- *read* calcium.

Page 451, line 14: *for* to Neuberg *read* to I. S. Neuberg.

Page 452, line 17: *for* sugar yeast *read* sugar by yeast.

Page 452, line 18: *for* reserved *read* critical.

Page 452, line 6 from bottom: *for* 20_2 *read* $2\,O_2$.

Page 452, line 5 from bottom: *for* O_2 *read* $2\,O_2$.

Page 452, line 2 from bottom: *for* (CH_2O) *read* CH_2O.

Page 453, line 18 from bottom: *for* reaction *read* reactions.

Page 453, line 4 from bottom: *for* discussed on page 464 *read* the course of which is known.

Page 454, line 18: *for* impossible *read* unlikely.

Page 454, line 24: *for* 21 *read* 2 *l*.

Page 454, line 11 from bottom: *for* manganate *read* permanganate.

Page 455, line 20: *for* 74 *read* 174.

Page 456, line 7: *delete* (see p. 464).

Page 456, line 12 from bottom: *for* & *read* and.

Page 460, line 8: *for* pyrophosphate *read* pyrophosphatase.

Page 460, line 19: *for* metaphosphate *read* metaphosphoric acid.

Page 461, line 17 from bottom: *for* page 000 *read* above.

Page 462, line 20: *for* $C(NHCH_3)\cdot CH_3$ *read* $CH(NHCH_3)\cdot CH_3$.

Page 463, line 12 from bottom: *insert comma after* p-toluylaldehyde.

Page 464, line 1: *after* yeast. *insert* i.e. $[\alpha]_D^{19} = 14.1°$ (unpublished).

Page 464, line 25: *for* (225) and refined (226) *read* and refined (225, 226).

Page 464, line 26: *for* Tomiyasu *read* Tomiyashu.

Page 464, line 27: *for* Reberg *read* Raborg *and for* 1 ml. *read* 0.8 µg. per ml.

Page 464, subhead: *change to* Morphology, Genetics, etc.

Page 465, line 1: *for* in intact *read* of intact.

Page 465, line 8 from bottom: *delete* S, .

Page 465, line 7 from bottom: *change sentence to read* Copulation of R cells is possible; R and S forms arise.

Page 467, line 9: *for* position, *read* position[1].

Page 467, line 19: *for* phenomenon[1] *read* phenomenon.

Page 467: *for* 4. Neuberg *read* 4. For literature see Neuberg.

Page 468, reference 20: *after* **21A** *insert* No. 4.

Page 468, reference 24: *after* **16A** *insert* Nos. 16 and 20.

Page 469, reference 51: *for* Pryde, T. *read* Pryde, J.

Page 469, reference 52: *for* (1942) *read* (1945).

Page 469, reference 68: *for* Zorkòrczy *read* Zorkorczy.

Page 469, reference 70: *for* T. *read* J.

Page 469, reference 74: *for* T. *read* J.

Page 469, reference 76: *delete Russian reference; retain Chem. Abstracts reference.*

Page 469, reference 79: *for* Vaidya *read* Vaydia; *for German reference insert instead* quoted by Sperber, reference 84.

Page 469, reference 84: *after* **21A** *insert* No. 3.

Page 469, reference 87: *for* T. *read* J.

Page 470, reference 96: *change to read* Pringsheim, H., and Kolodny, S., *Ber. deut. chem. Ges.,* 59, 1135 (1926).

Page 470, reference 99: *for* 2466 *read* 2486.

Page 470, reference 103: *for* T. *read* J.

Page 470, reference 115: *for* T. *read* J.

Page 472, reference 174: *after* **14B** *insert* Nos. 13 and 29.

Page 472, reference 186: *after* **15A** *insert* No. 12.

Page 472, reference 196: *for* Biol. *read* Geol.; *after* **16B** *insert* No. 16.

Page 472, reference 201: *for* Biol. *read* Geol.; *after* **16A** *insert* No. 5.

Page 472, reference 204: *change to* Cori, C. F., and Cori, G. T., *Ann. Rev. Biochem.*, **10**, 151 (1941).

Page 472: *change present reference* 204 *to* 204a.

Page 473, reference 206: *for* Myrbäch *read* Myrbäck.

Page 473, reference 222: *change journal citation* **197**, 257 (1928); **201**, 206 (1928).

Page 474, reference 259: *for* mikrobiol. *read* Mikrobiol.

Page 474, reference 263: *for* Hongo *read* Hongō.

Page 474, reference 273: *for* Lindgren, G., *read* Lindegren, G.

Page 474, reference 278: *for* Custers, M. T. T., *read* Custers, M. T. J.